

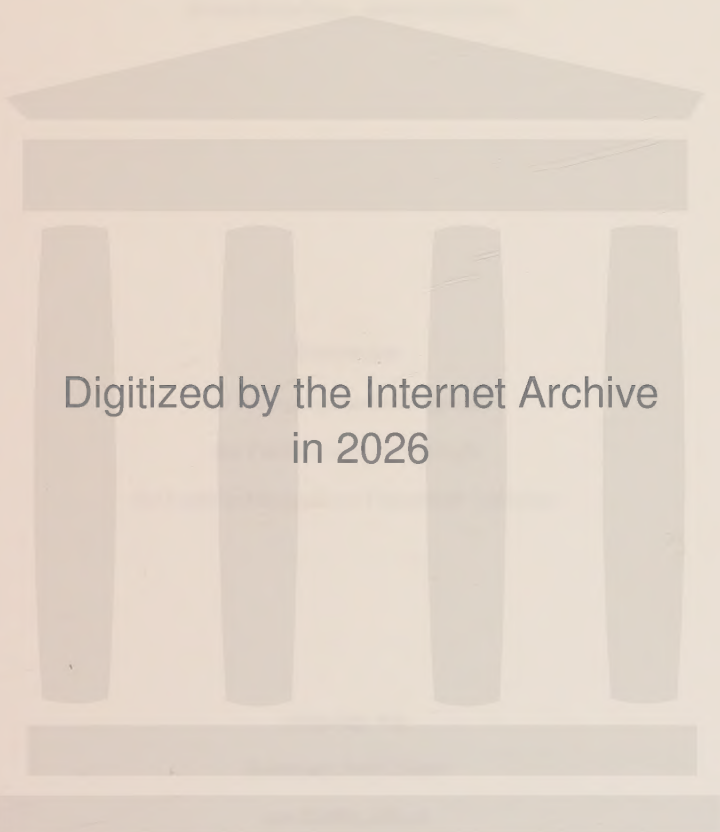
**Biogenesis of cytochrome c  
from *Neurospora crassa*:  
Characterization  
of mitochondrial targeting  
and translocation mechanisms**

Rosemary Anne Stuart

Disbui. München

1989





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**Biogenesis of cytochrome *c* from *Neurospora crassa*:  
Characterization of mitochondrial targeting and  
translocation mechanisms**

Dissertation

zur Erlangung des Doktorgrades

des Fachbereichs für Biologie

der Ludwig-Maximilians-Universität München

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Mai 1989

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Tag der mündlichen Prüfung: 24. 7. 1989

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For Mum and Dad



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## 1. INTRODUCTION

Eucaryotic cells, in contrast to procaryotic cells are divided into a number of compartments, each surrounded by one or more membranes. The major cellular compartments common to most eucaryotic cells hence are the nucleus, the endoplasmic reticulum, the golgi apparatus, mitochondria, peroxisomes, lysosomes and in the case of plant cells, chloroplasts. As a consequence of creating this compartmentalisation (i.e. organelles), eucaryotic cells have evolved a host of new capabilities compared with simple procaryotic systems. Each compartment contains a unique set of proteins that enables it to carry out specific functions. It is essential, therefore, for the maintenance and development of the cell that proteins destined for specific compartments are accurately and efficiently delivered to them. Organelle membranes must not only have devices to specifically recognize proteins which are destined for the compartment which they enclose, but must also have mechanisms to translocate these proteins across lipid membranes. The uniqueness of each type of organelle is thus maintained by processes in the cell which sort and deliver the individual components in a highly specific manner. This present study is concerned with the targeting of mitochondrial precursor proteins and their recognition by the mitochondria. The major emphasis will be placed on one particular protein, cytochrome *c*. The aim of the present Introduction is to highlight some salient features of protein translocation across diverse biological membranes and then to focus on the mechanism of protein import into mitochondria and to review our current knowledge in this field.

With the exception of the very small percentage of proteins encoded by the mitochondrial and chloroplast genomes, all protein synthesis begins with the formation of polysomes in the cytosol. Two mechanisms of protein translocation exist in eucaryotic cells which differ from each other on the basis of whether protein synthesis and translocation across biological membranes are temporally separated or not. In one mechanism, although synthesis starts on soluble polysomes, membrane choice and translocation are events coupled to translation (co-translational translocation). The other mechanism is called post-translational translocation, and is where the precursor proteins are synthesized on free polysomes and are transported to their target membrane or organelle after protein

synthesis of the polypeptide is completed. In general, most proteins destined for the extracellular space, the plasma membrane or lysosomes are all initially co-translationally inserted into or translocated across the endoplasmic reticulum (ER) membrane and are subsequently transported to their final destinations via the golgi complex. However, in contrast to proteins initially targeted to the ER, those proteins destined for the nucleus, peroxisomes, mitochondria and chloroplasts, can be translocated into these organelles following complete polypeptide synthesis and release into the cytosol, i.e. in a post-translational manner (de Robertis, 1983; Hallermayer *et al.*, 1977; Ellis *et al.*, 1980).

Protein targeting to all membranes, whether they be procaryotic or eucaryotic, share many salient features, the most popular of which at the moment is the notion of correct/proper conformation of precursor proteins so that they may be "translocation competent" (for reviews see Rothman & Kornberg, 1986; Zimmermann & Meyer, 1986; Meyer, 1988). A precursor protein is considered to have a translocation-competent conformation when it has an unfolded structure rather than when it has adopted the "foldedness" of its mature counterpart. A translocation-competent conformation is correlated therefore, with a certain openness of structure that is characterized by increased sensitivity to proteolytic digestion (Randall & Hardy, 1986; Pfanner *et al.*, 1987b). The fact that the precursor proteins must have this proper conformation in order to be translocated across a lipid bilayer is the central theme running through a range of present studies including, protein transport across the bacterial plasma membrane, the endoplasmic reticulum and import into mitochondria. Thus before going into detail of how cytoplasmically-synthesized proteins are imported into mitochondria, as an example of post-translational translocation, it seems pertinent to briefly review the recent exciting developments concerning the importance of precursor protein conformation in membrane translocation, as it is a central dogma common to all procaryotic and eucaryotic protein transport systems.

The role of cytosolic cofactors in keeping proteins in a correct conformation for translocation has been most elegantly demonstrated by Wickner and co-workers in their study of *E. coli* pro-OmpA assembly into inverted plasma membrane vesicles. Pro-OmpA that was over-expressed in *E. coli*, and treated with urea, to unfold the protein (followed by rapid dilution) (Crooke & Wickner 1987; Crooke *et al.*, 1988) was only

transiently translocation-competent; as in the absence of urea refolding of the precursor accompanied by the loss of ability to become translocated took place unless removal of urea by dialysis was performed in the presence of cytosol (Crooke & Wickner, 1987). A component of the cytosol was purified, which alone could account for this conformation stabilizing activity, namely, the trigger factor, a protein of 63,000 daltons (Crooke *et al.*, 1988). Trigger factor not only binds to pro-OmpA in a 1:1 stoichiometric ratio, but it could also bind to the large subunit of ribosomes and the complex of pro-OmpA:trigger factor can bind to specific sites on the surface of the plasma membrane (Lill *et al.*, 1988). These findings lead Wickner to propose a model of trigger factor cyclic action, which bears a number of similarities to the SRP-docking protein model for proteins targeted to the ER (Walter & Blobel, 1982; Meyer *et al.*, 1982; Gilmore *et al.*, 1982). Trigger factor binds to nascent polypeptide chains, most probably co-translationally *in vivo*, and by doing so ensures that the newly-synthesized precursor does not fold into a conformation incompetent for translocation. This complex once formed, is targeted to receptor sites on the plasma membrane from where pro-OmpA is released from trigger factor (probably in an ATP-dependent reaction) and it pursues its passage across the lipid bilayer. Trigger factor itself is released into the cytosol where it returns to a ribosome and awaits another nascent chain to bind to (Lill *et al.*, 1988).

More interestingly, however, not only does this trigger-factor cycle resemble that of the "SRP-cycle", but SRP is functionally interchangeable for the trigger factor (Crooke *et al.*, 1988; Sanz & Meyer, 1988). It is important to emphasize that SRP, like trigger factor, cannot act post-translationally, i.e. once the polypeptide has folded; it may therefore be concluded that they do not possess an unfoldase function, thus suggesting that *in vivo* trigger factor acts co-translationally binding to nascent chains during their synthesis and prior to their folding.

The virtues and benefits of co-translational translocation therefore seem very attractive, SRP prevents unfavorable folding of precursors while at the same time targets them to their correct membrane via its action with the docking protein. The question thus arises as to how precursor proteins which are imported post-translationally, manage to keep themselves translocation competent until such a time they find their membrane of

destination. It has been previously demonstrated that folded precursor proteins cannot be imported across the mitochondrial membranes (Eilers & Schatz, 1986; Chen & Douglas, 1987). On the other hand, hydrolysis of nucleoside triphosphates (NTPs) is also necessary for mitochondrial protein import (Pfanner & Neupert, 1986; Pfanner *et al.*, 1987b). It was demonstrated that the sensitivity of precursor proteins in reticulocyte lysate to non-specific proteases is greater in the presence of NTPs (Pfanner *et al.*, 1987b). Thus it was postulated that the hydrolysis of NTPs is somehow involved in modulating the folding of precursors in the cytosol. How NTPs are involved in conferring a conformation on a precursor that is competent for import was the subject of much recent attention. The answer to this problem may lie in a group of proteins known as heat shock proteins. Prokaryotic and eukaryotic cells, in response to heat shock proteins, the most abundant of which form two families known as hsp70 and hsp90 and are conserved among a diverse range of prokaryotic and eukaryotic organisms (Pelham, 1986, 1988). Members of both families are constitutively expressed and a subset of the hsp70 family are required for cell viability (Craig & Jacobsen, 1984). The ability of heat shock proteins in times of stress to bind to exposed hydrophobic areas of denatured proteins and thereby promote disaggregation, has been well documented (i.e. in the case of nucleoli protection in heat shocked mammalian cells) (Pelham, 1984; Lewis & Pelham, 1985; Welch & Suhan, 1986). Following heat shock, hsp70 frees itself from its substrate in an ATP-dependent reaction and the substrate subsequently refolds (Pelham, 1986; 1988). In a review article, Pelham postulated that hsp70 proteins, because they possess the ability to interact with hydrophobic regions of proteins can function by binding to nascent polypeptide chains, which by virtue of their nature, may also be defined as denatured, and in doing so prevent their possibility of folding into translocation incompetent forms (Pelham, 1986). Pelham's postulation was soon shown to be indeed correct, as recently two groups, namely those of Schekman and of Blobel, independently showed in post-translational assays that the translocation not only of prepro  $\alpha$ -factor across microsomal membranes in yeast, but also import of the  $F_1$ -ATPase subunit  $\beta$  into mitochondria also required the involvement of hsp70 proteins (Deshaies *et al.*, 1988 a, b; Chirico *et al.*, 1988). This work also presented for the first time evidence for a common component linking protein translocation systems of the ER and mitochondria. It was concluded from

these studies that the heat shock protein was acting in a NTP-dependent manner to maintain the various precursor proteins in an translocation-competent conformation (Deshaies *et al.*, 1988b). It would appear, in other words that heat shock proteins, like trigger factor and SRP, may act in a preventative manner by inhibiting folding rather than coming to the rescue of an already folded protein by unfolding them. It is very attractive to speculate that heat shock proteins play such an essential role in the import of all post-translationally imported proteins. By binding in a co-translational manner, they would prevent folding and aggregation and thereby deliver the bound precursor to the surface of their destination membrane in a translocation-competent form. Release of the heat shock protein from its bound state would require ATP, as previously described for hsp activity (Pelham, 1986). In this respect it is interesting to note that all mitochondrial precursor proteins which require an unfolded conformation to be translocation competent, display a dependence on ATP for initial membrane insertion (Pfanner & Neupert, 1986; Pfanner *et al.*, 1987b). Cytochrome *c* import, on the other hand, requires that the precursor has a specific folded conformation and is ATP-independent (Nicholson & Neupert, in preparation). It would be interesting to investigate if cytochrome *c* import is also consequently hsp-independent.

As mentioned previously most proteins targeted to the ER are translocated in a co-translational manner, however, small precursor proteins can be imported post-translationally and in a SRP-docking protein independent manner (Müller & Zimmermann, 1987, 1988; Schlenstedt & Zimmermann, 1987; Wiech *et al.*, 1987). Interestingly, it has been recently demonstrated that translocation of these precursor proteins across the microsomal membrane is however, dependent on hsp70 proteins and the presence of an ATPase activity in the cytosol (Zimmermann *et al.*, 1988). Another role of heat shock proteins, consistent with the theme of folding and translocation-competence has been recently described. A yeast mutant, *mif4*, was selected on the basis that it was deficient in assembly of imported mitochondrial proteins (Cheng *et al.*, 1989). The *Mif4* gene codes for a heat shock protein hsp60, which is structurally related to the *E. coli* groEL protein and to the Rubisco subunit binding protein in chloroplasts. It was postulated that hsp60 acts as a chaperonin (Mc Mullin & Hallberg, 1988; Hemmingsen *et*

*al.*, 1988; Reading *et al.*, 1989) which mediates the correct folding and assembly of proteins imported initially into the mitochondrial matrix.

As mentioned previously, the mitochondrial genome codes for a small percentage of the mitochondria's total protein complement, the remainder are specified by nuclear genes (Neupert & Schatz, 1981). The nuclear encoded proteins are imported into mitochondria along complex pathways. These import pathways involve a series of steps which act in concert to efficiently and specifically direct proteins to their correct intracellular and intramitochondrial location. Many of these steps are common to a large number of mitochondrial protein constituents (for reviews see Nicholson & Neupert, 1988; Pfanner *et al.*, 1988b; Hartl *et al.*, 1989). In general mitochondrial proteins are synthesized on free ribosomes as precursors, containing N-terminal extension sequences, and are released into the cytoplasm (Hallermayer *et al.*, 1977). These precursors are subsequently imported into one of the four mitochondrial subcompartments, namely, outer membrane, intermembrane space, inner membrane or matrix. Although binding and import does not depend on a coupled translational event, as mentioned above, it is still quite possible that under certain physiological conditions *in vivo*, mitochondrial precursors could be imported in a co-translational manner (Schleyer & Neupert, 1985).

The N-terminal prepiece present on most mitochondrial precursor proteins contains the necessary information to target these proteins to mitochondria (Hurt *et al.*, 1984 a, b; Douglas *et al.*, 1984; Horwich *et al.*, 1985, 1986; Emr *et al.*, 1986; Keng *et al.*, 1986; Vassarotti *et al.*, 1987, van Loon & Young, 1986). As will be discussed later these presequences can also play an important role in subsequent intramitochondrial sorting (van Loon *et al.*, 1986; Hartl *et al.*, 1986, 1987). It is important to note, however, that some precursor proteins do not contain N-terminal extensions. For example, the outer membrane proteins porin and a 70 kD protein from yeast (Mihara *et al.*, 1985; Kleene *et al.*, 1987; Kiezman *et al.*, 1983a; Hase *et al.*, 1984) and cytochrome *c* of the intermembrane space (Zimmermann *et al.*, 1981; Stuart *et al.*, 1987) are not proteolytically processed upon import. Clearly specific targeting information for these precursor proteins must reside within the mature part of the polypeptide itself, although it is not known whether such targeting information operates on the level of the primary

structure or if it is independent of a critical conformational arrangement of the protein (Stuart *et al.*, 1987; Nicholson *et al.*, 1987b, and this work).

Proteinaceous components (import receptors) on the surface of the outer membrane are involved in the initial recognition process of the precursor proteins (Zwizinski *et al.*, 1983, 1984; Riezman *et al.*, 1983b; Pfaller & Neupert, 1987; Pfaller *et al.*, 1988). The high-affinity binding of the precursor of porin to mitochondria has been studied in detail (Pfaller & Neupert, 1987). Scatchard analysis has indicated the existence of high-affinity binding sites present at 5-10 pmol/mg of mitochondrial protein and having an apparent  $K_a$  of  $(1.5) \times 10^8 \text{ M}^{-1}$ . The binding of porin to mitochondria, like for most precursor proteins, was sensitive to pretreatment of the mitochondria with very low concentrations of trypsin, indicating that these binding sites are of a proteinaceous nature. It has been recently reported that precursor proteins that are bound specifically to receptors on the mitochondrial surface are subsequently transferred to a component in the outer membrane called GIP "general insertion protein" (Pfanner *et al.*, 1988a; Pfaller *et al.*, 1989). Transfer of precursors to this common insertion site can be competed for by water-soluble porin (the precursor form of porin, produced by chemically denaturing isolated porin from mitochondria) (Pfaller *et al.*, 1985). Competition studies with water-soluble porin can therefore be used to determine whether GIP is involved in the import of other precursor proteins or not. It was thus determined that all precursors studied to date, with the exception of cytochrome *c*, required the presence of an active GIP to mediate their insertion into the mitochondrial membranes (Pfaller *et al.*, 1989).

Transport of precursors across the two mitochondrial membranes occurs in a single step at contact sites between the outer and inner membrane (Schleyer & Neupert, 1985; Schwaiger *et al.*, 1987). The insertion into and translocation across the outer and inner membranes are dependent on an electrochemical potential ( $\Delta\Psi$ ) across the inner membrane (Schleyer *et al.*, 1982; Gasser *et al.*, 1982a; Pfanner & Neupert, 1985). It is proposed that the electrophoretic driving force on the positively charged parts of the precursor protein serves to mediate penetration into or through the inner membrane (Pfanner & Neupert, 1986; Roise *et al.*, 1986).

As previously described in detail, the role of nucleoside triphosphates (NTPs) in the transport of proteins into the mitochondrion has also been investigated (Pfanner & Neupert, 1986; Pfanner *et al.*, 1987b; Chen & Douglas, 1987; Eilers *et al.*, 1987, Verner & Schatz, 1987; Eilers & Schatz, 1988). As concluded above, the requirement of NTPs is linked with processes which are involved with conferring an import competent conformation on the precursor during the processes of recognition and binding to the mitochondrion. During or shortly following the translocation process, precursor proteins with N-terminal presequences are proteolytically processed by a metal-dependent processing peptidase located in the matrix (Böhni *et al.*, 1980; Miura *et al.*, 1982; Conboy *et al.*, 1982; McAda & Douglas, 1982; Schmidt *et al.*, 1984; Hawlitschek *et al.*, 1988; Witte *et al.*, 1988; Pollock *et al.*, 1988). The processing peptidase has recently been purified from *Neurospora crassa* (Hawlitschek *et al.*, 1988) and was shown to consist of two polypeptides of 57kD and 52kD apparent molecular mass. The catalytic activity is associated with the 57 kD component, termed the matrix processing peptidase (MPP). The other component, termed the processing enhancing protein (PEP), is required as an "assistant protein", and functions to increase the catalytic rate and specificity of the processing reaction. The MPP and PEP components have now been cloned and derived amino acid sequence data revealed that MPP bears homology to PEP, thus suggesting that the respective genes are of common evolutionary origin (Hawlitschek *et al.*, 1988; Pollock *et al.*, 1988).

Once specifically directed to mitochondria, imported precursor proteins must then be correctly sorted to their functional intramitochondrial location. Generally sorting of precursors is accomplished by direct transfer to their correct mitochondrial subcompartments. In some cases, however sorting occurs by a more sophisticated mechanism (for example, the precursors of the Fe/S protein and cytochrome  $c_1$  of the ubiquinol-cytochrome  $c$  reductase complex and cytochrome  $b_2$  and (Hartl *et al.*, 1986, 1987), all of which function in the intermembrane space and contain a presequence which is processed in two distinct steps). In these cases, the precursors are first imported completely into the matrix where the first part of the presequence is removed by the processing peptidase to generate an intermediate-size product. This is then redirected back across the inner membrane to the intermembrane space where the second half of the

presequence is proteolytically removed. Contrary to the "stop-transfer" model (van Loon & Schatz, 1987; van Loon *et al.*, 1987), hydrophobic stretches of amino acid in the second half of the prepiece appear to act as transport signals to direct export from the matrix into the intermembrane space. (Hartl *et al.*, 1986, 1987). This process was termed "conservative sorting" for the following reasons. Following the endosymbiotic event, evolutionary gene transfer from the bacterial endosymbiont to the host cell nucleus occurred. Thus the newly formed eucaryotic cell had to evolve a whole spectrum of new mechanisms, outlined above, to return gene products to their functional location in the mitochondrion. Rather than completely reconstruct the means by which a cytoplasmically synthesized mitochondrial protein was imported and assembled, the import pathways evolved so as to make use of evolutionary remnants of pre-existing "ancestral assembly pathways" of the bacterial endosymbiont. Hence some precursors are initially imported into the mitochondrial matrix (a compartment comparable with the bacterial cytosol) from there they are "exported" across the inner membrane along their ancestral pathways, this process is therefore termed "conservative sorting".

On the other hand, not all mitochondrial precursor proteins follow this elaborate pathway. It is interesting in this regard to compare the import pathways of the two *c*-type cytochromes in mitochondria, namely of cytochrome *c* and  $c_1$ . They are markedly different. Cytochrome  $c_1$  is initially synthesized in the cytosol as a precursor protein ( $pC_1$ ) with a long amino-terminal prepiece that is processed in two steps upon import into mitochondria (Teintze *et al.*, 1982; Schleyer & Neupert, 1985; Gasser *et al.*, 1982b; Sadler *et al.*, 1984; Römisch *et al.*, 1987). The presequence consists of two domains, the first a positively charged "matrix-targeting" domain and the second, a rather hydrophobic sequence (van Loon *et al.*, 1987). The precursor initially binds to protease-sensitive receptors on the mitochondrial outer surface. The  $pC_1$  inserts into the outer membrane, a process requiring the general insertion protein (GIP) (Pfaller *et al.*, 1989; and this work) and is then imported via contact sites (Schleyer *et al.*, 1982) into the mitochondrial matrix in a membrane potential-dependent fashion. In the matrix it is processed to its intermediate form ( $iC_1$ ) by the processing peptidase (MPP). The second domain of the presequence (which bears significant similarity to the bacterial cytochrome  $c_2$  and  $c_1$  leader sequences (see Figure 37, Discussion) directs the "export" of the protein along its

"ancestral transport pathway" across the inner membrane (Hartl *et al.*, 1986, 1987).  $iC_1$  thereby becomes exposed to the intermembrane space while being anchored to the inner membrane via its carboxy terminus (Wakabayashi *et al.*, 1980; Li *et al.*, 1981). The covalent attachment of heme to  $iC_1$  occurs in a reaction catalysed by cytochrome  $c_1$  heme lyase, a reaction which is dependent on the presence of NADH and flavin nucleotides (Nicholson *et al.*, 1989). Once this reaction has taken place, but not before, the second proteolytic processing of cytochrome  $c_1$  occurs resulting in the production of the mature-size cytochrome  $c_1$  ( $mC_1$ ) (Teintze *et al.*, 1982; Nicholson *et al.*, 1989).

Although mitochondrial cytochromes  $c$  and  $c_1$  share a number of similarities with regard to function, submitochondrial location and prosthetic group, the import pathway of cytochrome  $c$  has evolved to be strikingly different from that of cytochrome  $c_1$ . Cytochrome  $c$  is synthesized as a precursor, known as apocytochrome  $c$ , which does not contain an amino-terminal prepiece and thus is not proteolytically processed upon import into mitochondria (Zimmermann *et al.*, 1981; Stewart *et al.*, 1971; Zitomer and Hall, 1976; Smith *et al.*, 1979; Matsuura *et al.*, 1981; Scarpulla *et al.*, 1981; Stuart *et al.*, 1987). No protease-sensitive components exist on the surface of mitochondria to mediate apocytochrome  $c$  binding and import (Nicholson *et al.*, 1988). Instead, apocytochrome  $c$  partially inserts into the outer mitochondrial membrane (Rietveld & de Kruijff, 1984; Rietveld *et al.*, 1985, 1986; Dumont & Richards, 1984; Berkhout *et al.*, 1987) where it is recognized and binds with high affinity to specific binding sites (Korb & Neupert, 1978; Hennig & Neupert, 1981; Hennig *et al.*, 1983; Nicholson *et al.*, 1987) in a complex which includes cytochrome  $c$  heme lyase (CCHL) (Nicholson *et al.*, 1988). CCHL is the enzyme responsible for the covalent attachment of heme to apocytochrome  $c$  and it displays a dependence on NADH and flavin nucleotides (Nicholson & Neupert, 1988). It is thought that cytochrome  $c$  heme lyase acts as a specific binding site *in lieu* of a surface receptor (Nicholson *et al.*, 1988). A mutant of *Neurospora crassa*, *cyt2-1*, whose mitochondria are deficient in CCHL activity, also are unable to bind apocytochrome  $c$  or produce holocytochrome  $c$  (Nargang *et al.*, 1988). This result therefore is consistent with the view that CCHL functions also as an initial binding site for apocytochrome  $c$ . Specifically-bound apocytochrome  $c$  remains accessible to externally added proteases until holocytochrome  $c$  formation has occurred. Hence it was concluded that holo-formation

was a prerequisite for import of apocytochrome *c* (Hennig & Neupert, 1983; Nicholson *et al.*, 1988; Dumont *et al.*, 1988; Nicholson & Neupert, 1989). Unlike all other mitochondrial precursors crossing the outer membrane, cytochrome *c* does not require a membrane potential for translocation (Zimmermann *et al.*, 1981; Pfanner & Neupert, 1985). Furthermore, in contrast to all other precursors studied so far, ATP is not required throughout the import process of cytochrome *c* (Nicholson & Neupert, in preparation, and this work), thus suggesting that a specific folded, rather than unfolded, conformation of apocytochrome *c* is required for binding and membrane insertion. Translocation across the outer membrane, directly into the intermembrane space is believed to be driven by the refolding of the polypeptide as a result of the covalent attachment of heme, a reaction catalysed by the enzyme CCHL (Hennig & Neupert, 1981; Nicholson *et al.*, 1988; Nicholson & Neupert, 1989).

## 2. Aims of the present study.

The presequences of mitochondrial precursor proteins contain the necessary information to correctly target them to mitochondria. Recognition of these precursor proteins is mediated by receptor proteins which exist on the surface of the mitochondria. The import of cytochrome *c*, however, is exceptional when compared with the general targeting mechanism displayed by other mitochondrial precursor proteins. The question thus arises as to how apocytochrome *c*, which does not contain a cleavable presequence, is targeted to mitochondria and how it binds with high affinity to mitochondria in the absence of surface receptors. The aim of this present study was to address the two aspects of this problem.

Initially, in order to investigate how apocytochrome *c* is specifically targeted to mitochondria, a mutant of *Neurospora crassa*, *cycl-1*, whose mitochondria are deficient in cytochrome *c*, was examined. Apocytochrome *c* is in fact synthesized in the mutant cells, but it is incompetent for import into mitochondria. To investigate specifically why the mutant cytochrome *c* precursor was import incompetent, it was necessary to obtain cDNA clones not only for the *cycl-1* cytochrome *c* but also for the wild-type cytochrome *c* so that they could be compared. Sequence studies of both clones were performed and the similarities and differences of the wild-type cytochrome *c* mRNA compared with those of the mutant mRNA, are discussed with reference to the targeting of apocytochrome *c* to mitochondria.

The import pathway of cytochrome *c* into mitochondria appears mechanistically simple in contrast to that of cytochrome *c*<sub>1</sub>. Although the bacterial ancestors of cytochrome *c* and *c*<sub>1</sub> are very similar with regard to leader sequences, assembly pathways, function and final location in the periplasm, a striking divergence in the evolution of the import pathways of their mitochondrial counterparts has occurred. The question thus arises as to how cytochrome *c* biogenesis has evolved to escape the "conservative sorting" pathway that cytochrome *c*<sub>1</sub> and other precursors must follow to gain access to the mitochondrial intermembrane space. Hence, it was investigated as to what special endogenous property the apocytochrome *c* polypeptide has, that enables it to discard all the classical features of mitochondrial protein import (precursor presequences, surface receptors, GIP-dependent

translocation) and take a completely unique import pathway into the mitochondria. In order to do this a series of fusion proteins between apocytochrome *c* and mitochondrial presequences were constructed. As a result of studying the mitochondrial import pathways of these fusion proteins, it was revealed that the presence of the apocytochrome *c* domain bestowed unique properties on the fusion proteins. The presence of a membrane-active domain (namely, apocytochrome *c*) enabled the fusion proteins to be imported into the mitochondrial matrix in a manner that was no longer dependent on either surface receptors, or GIP-mediated membrane insertion or on NTP-mediated precursor unfolding. The importance of these results are discussed with reference not only to unique features of the apocytochrome *c* precursor protein, but also to how these special attributes of the precursor molecule may have played a significant role in the evolution of the cytochrome *c* import pathway.

### 3. MATERIALS AND METHODS

#### 3.1 Strains of *Escherichia coli* and *Neurospora crassa* used

*Escherichia coli* strain 5K (Hubacek & Glover, 1970) was used as a host for the cDNA and genomic libraries cloned into the plasmid pBR322 (Bolivar *et al.*, 1977; Sutcliffe, 1978). *E. coli* strains HB101 (Kedes *et al.*, 1975) and DH1 (Low, 1968) were used as hosts for the plasmids pDS5 (Stueber *et al.*, 1984), pUC19 (Vierira & Messing, 1982) and pGEM3,4 (Melton *et al.*, 1984; Promega). *E. coli* cells WK6 and WK6*mut*s (Levinson *et al.*, 1984) were used as hosts for the plasmid pMa/c5-8 (Fritz *et al.*, 1986).

Strains of *Neurospora crassa* used in this study were the wild-type strains 74A (Fungal Stock Center No. 262) and two derivative strains, namely, *cyc1-1*, obtained from H. Bertrand, Regina, Canada and *cyt2-1 pan-2a* strain obtained from F. E. Nargang, Alberta, Canada.

#### 3.2 Preparation of total polyadenylated RNA (Michel *et al.*, 1979)

Total RNA was initially isolated from *Neurospora crassa* hyphae as described by Michel *et al.*, 1979. Hyphae yielded from a 16 l culture were frozen in liquid nitrogen and were ground to a fine powder in a mortar and pestle precooled with liquid nitrogen. Extra N<sub>2</sub> was added throughout the grinding procedure to keep the hyphae frozen. The resulting powder was divided into 50 g portions and were shaken with 150 g sterile quartz sand and 200 ml extraction medium I +II (1:1) at room temperature for 20 min.

Extraction medium I (Leaver and Ingle, 1971): 10 mM Tris-HCl pH 8.2, 50 mM KCl, 6 % (w/v) p-aminosalicylate, 2 % (w/v) tri(isopropyl)naptalinsulphonate.

Extraction medium II: 50 % (v/v) phenol medium, 49 % (v/v) chloroform, 1% (v/v) isoamylalcohol.

*Phenol medium* (Kirby *et al.*, 1968):

500 ml phenol saturated with Tris-HCl pH 8.2, 75 ml freshly distilled m-cresol, 0.5 g 8-hydroxyquinoline.

The organic and aqueous phases were separated by centrifugation at 11,000 x g for 15 min (Beckmann JA-10 rotor). The aqueous phase was re-extracted twice with equivalent volumes of chloroform/isoamylalcohol (49:1 v/v). Two volumes of ethanol (Merck, p.A.) were added and the RNA was allowed to precipitate overnight at -20 °C. The RNA was collected by centrifugation and washed once with 66 % ethanol and dried in a vacuum pump. The RNA was subsequently resolubilized in binding buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 100 mM NaCl, at a concentration of approximately 100 O.D./ml (260 nm).

A poly(U)-cellulose column (3.2.1) was employed to purify the poly(A)<sup>+</sup> mRNA from the total extracted RNA (Sheldon *et al.*, 1972). The column (1.2 x 2.0 cm) was equilibrated with the above mentioned binding buffer and the RNA fraction was applied to the column. The column was then washed with the binding buffer (flow rate 1 ml/min) until the O.D. at 280 nm of the eluate reached zero. Elution of the bound poly(A)<sup>+</sup> mRNA was achieved by washing the column with the following elution buffer 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 90 % (v/v) formamide. Poly(A)<sup>+</sup> mRNA was precipitated at -20 °C overnight by adjusting the salt concentration of the resulting eluate to 0.2 M sodium acetate and by the addition of two volumes of ethanol. The poly(A)<sup>+</sup> mRNA was recovered by centrifugation, washed three times with 70 % ethanol, vacuum dried and resolubilized in sterile water and stored at -80 °C in portions.

### 3.2.1 Preparation of the Poly(U)-cellulose column (Sheldon *et al.*, 1972)

Cellulose powder (15 g, Schleicher & Schüll) was serially washed with 200 ml ethanol, 100 ml 1M HCl, 500 ml sterile water and 100 ml ethanol. The powder was dried and was carefully mixed with 15 ml of poly(U) (8 mg/ml). Following lyophilization, the resulting powder was resuspended in 150 ml ethanol, spread thinly on a flat glass dish and was subjected to 6 x 5 min U.V. illumination periods (10 W lamp, 10 cm distance). The cellulose was thoroughly washed with water, followed by elution buffer, water and finally ethanol. The poly(U)-cellulose powder was dried and stored at -20 °C until used. Approximately 3g of poly(U)-cellulose were used for 750 mg of total RNA.

### 3.3 Synthesis and cloning of the *N. crassa* cDNA library

#### 3.3.1 cDNA synthesis

Polyadenylated RNA was isolated from *N. crassa* wild-type (74 A) and from a mutant *cycl-1*, and cDNA synthesis was performed for both as described by Gubler & Hoffman (1983) with certain modifications.

*Synthesis of the first strand* : Poly(A)<sup>+</sup> mRNA (10 µg) was dissolved in 20 µl H<sub>2</sub>O and incubated for 5 min at 65 °C and was then transferred immediately to ice. The following were added to the RNA (10 µl), and in the following order: 2.5 µl Tris-HCl pH 8.3 (1M), 5 µl KCl (1M), 4 µl MgCl<sub>2</sub> (0.25 M), 5 µl oligo-dT<sub>12-18</sub> (1 mg/ml), 2.5 µl DTT (200 mM), 5 µl of each dATP, dGTP, dTTP (10 mM) , 2.5 µl dCTP (10 mM) and 3 µl [ $\alpha$ -<sup>32</sup>P]dCTP (10 mCi/ml, 3000 Ci mmol). RNasin (1µl of 30 U/µl) and 3.2 µl AMV reverse transcriptase (50 U) (Life Science). Incubation was at 42 °C for 1 h. The reaction was stopped by adding 2.5 µl EDTA (500 mM) and was phenol extracted. The aqueous phase was treated with half the volume of 7.5 M ammonium acetate and with 2-3 volumes of ethanol and stored at -20 °C overnight. RNA/DNA was collected by centrifugation at 17,400 x g for 15 min (Beckman JA-20 rotor), washed with 70 % ethanol, vacuum dried and finally resuspended in 61 µl H<sub>2</sub>O.

*Second-strand synthesis*: To 50 µl of the RNA/DNA the following were added, 10 µl 10 x reaction buffer (200 mM Tris-HCl pH 7.5, 50 mM MgCl<sub>2</sub>, 100 mM ammonium sulphate, 1M KCl), 2 µl of each dATP, dCTP, dGTP, dTTP (2 mM), 10 µl βNAD<sup>+</sup> (1.4 mM), 4 µl RNase H (1 unit/ml, B.R.L.) and 6 µl DNA polymerase I (5 units/µl, Boehringer). Incubation was performed at 13 °C for 1 h followed by 22 °C for 1 h. The reaction was stopped by the addition of 4 µl EDTA (0.5 M) and was phenolized. The resulting aqueous phase was treated with an equal volume of 5 M ammonium acetate and two volumes of ethanol and stored at -20 °C overnight. The cDNA was recovered by centrifugation, washed in 95 % ethanol, dried and resuspended in 50 µl buffer D (3.6.1).

#### 3.3.2 dC/dG-tailing and annealing

The plasmid pBR322 was cut with *Pst*I (3.12.1) and purified from 0.8% (w/v) agarose gel by electroelution (3.10.1). Tailing of this vector with 15-20 residues of dG was performed as described by Hoeijmakers *et al.*, (1980). Tailing of the double-stranded

cDNA was performed as follows: 150 ng cDNA was tailed in a buffer containing 140 mM cacodylate, 60 mM Tris-HCl pH 7.6, 1 mM  $\text{CoCl}_2$ , 0.1 mM dCTP, in a total volume of 100  $\mu\text{l}$ . After a preincubation for 5 min at 37 °C, terminal deoxynucleotidyl transferase (33U, Pharmacia) was added and the reaction was performed for 3 min at 37 °C, terminated by the addition of 2.5  $\mu\text{l}$  EDTA (0.5 M) pH 8, heated to 65 °C for 10 min and used without further purification. dG-tailed pBR322 (50 ng) was annealed to 10 ng dC-tailed cDNA in 100  $\mu\text{l}$  of 100 mM, 1 mM EDTA, 10 mM Tris-HCl pH 7.8, by heating it to 68 °C for 5 min and cooling slowly overnight to room temperature.

### 3.3.3 Transformation of cDNA into *E. coli*

The annealing mixture (10  $\mu\text{l}$ ) was used to transform 200  $\mu\text{l}$  of freshly prepared competent *E. coli* 5K cells (3.11). The entire transformation mixture was plated out onto agar master plates containing tetracycline (5 mg/l) and incubated at 37 °C overnight. This resulted in approximately 1000 colonies per plate.

## 3.4 Construction of the *N. crassa* genomic DNA library

Genomic DNA was isolated from *N. crassa* wild-type (74 A) and the mutant *cyc1-1* by Dr M. Tropschug. A *N. crassa* (74 A) genomic library was constructed in the following manner. Genomic DNA was partly digested with *Sau3A* and 4-6 kb fragments were cloned into *Bam*I cleaved pBR322. The recombinant plasmids were transformed into *E. coli* 5K (3.11).

## 3.5 Colony hybridization (Grunstein & Hogness, 1975; Maniatis *et al.*, 1985)

A numbered Biotodyne filter was laid onto the surface of the master plate containing the bacterial colonies to be screened and was marked with a syringe needle, thereby identically marking both filter and master plates. The filters were then peeled off and placed onto fresh agar plates (containing tetracycline, 5 mg/l) with the colonies facing upwards. Both the master plates and filter plates were incubated at 37 °C for 4-6 h. Master plates were sealed and stored at 4 °C. The filters were removed and placed on

fresh plates, this time containing chloramphenicol (150 mg/l) and were incubated at 37 °C overnight.

In order to lyse the colonies and to fix the liberated DNA to the filter, the following procedure was carried out. Filters were placed individually in petri dish lids containing 3 ml NaOH (0.5 M) for 5 min. They were removed, dried briefly on Whatman filter paper and then once again denatured in 3 ml NaOH (0.5M). Filters were then neutralized by repeating the above procedure twice using 3 ml of Tris/acetate pH 7.5 (1 M) and then once with 0.5 M Tris-HCl, 1.5 M NaCl. Filters were then placed on 2.5 ml proteinase K (100 µg/ml in 1 x SSC (0.15 M NaCl, 0.015 M Na-citrate), 0.5 % (w/v) SDS) and incubated for 1 h at 42 °C. Filters were allowed to air dry by placing them on Whatman filter paper, and then were baked for 2 h at 80 °C.

Prewashing of the filters to remove any absorbed medium, agar fragments or bacterial debris was performed by gently shaking filters at 65 °C in 500 ml of 3 x SSC, 0.1 % (w/v) SDS, overnight, with frequent buffer changes.

### 3.5.1 Hybridization of filters with [<sup>32</sup>P]-labelled cDNA

Filters were initially pre-hybridized by incubating them at 65 °C for 30 min in 200 ml of hybridization buffer (2 x SSC, 0.1 % (w/v) SDS, 0.2 % (w/v) BSA, 0.2 % (w/v) Ficoll 400, 0.2 % (w/v) polyvinylpyrrolidone). The buffer was then discarded and fresh hybridization buffer containing 2 ml salmon sperm DNA (2 mg/ml), which had been previously denatured by heating to 95 °C for 5 min and then quickly cooled in ice-water, was added. Incubation at 65 °C was continued for a further 5-15 h. Fresh hybridization buffer plus salmon sperm DNA was added, in combination this time with nick-translated [<sup>32</sup>P]-labelled DNA (3.12.7) which had also been denatured by heating to 95 °C and rapid cooling on ice. Hybridization was performed overnight again at 65 °C.

A 280-bp apocytochrome *c* cDNA insert (A12/20) was identified (M. Tropschug & W. Neupert) by hybridization selection of mRNA (Alwin *et al.*, 1977; Viebrock *et al.*, 1982), subsequent translation in a reticulocyte lysate (3.15.3) and immunoprecipitation of the [<sup>35</sup>S]-labelled product with a cytochrome *c*-specific antibody (3.23). This insert, A12/20, was nick-translated and used to identify full-length wild-type cDNA inserts. Subsequent

full-length wild-type cytochrome *c* cDNA inserts were used to screen *cyc1-1* mutant cDNA and wild-type genomic gene banks.

Following hybridization, filters were washed as follows: initially at room temperature for 5 min, twice with hybridization buffer, twice with 2 x SSC, 0.1% (w/v) SDS, and then twice with 2 x SSC. Filters were then washed twice for 10 min with 0.2 x SSC at 65 °C and then allowed to air dry. Hybridization of the [<sup>32</sup>P]-labelled probe to recombinant clones containing cytochrome *c*-specific cDNA inserts were monitored by autoradiography. Such single positive clones were recovered from the master plates and were streaked once again onto agar plates containing tetracycline and were allowed to grow overnight at 37 °C. These colonies were screened once again by colony hybridization in order to eliminate "false positives" identified by the first screen. Recombinant plasmids were purified from positively identified colonies as outlined below (3.6.1).

### 3.6 Isolation of plasmid DNA

#### 3.6.1 *Small scale plasmid DNA isolation for analytical purposes*

"Mini preparations" of plasmid DNA were performed according to the method of Birnboim and Doly (1979), which was modified for direct sequencing of the DNA, to include a RNase treatment and a polyethylene glycol precipitation of the plasmid DNA.

5 ml of LB-medium (10 g Bacto-Tryptone, 5 g Yeast extract, 10 g NaCl per 1 l H<sub>2</sub>O) containing the appropriate antibiotic, was inoculated with a single bacterial colony and incubated overnight at 37 °C with vigorous agitation (165 rpm). A portion of the culture (0.5 ml) was removed and mixed with an equal volume of 88% (w/v) glycerine and stored at -20 °C. Such cell cultures served as stock cultures for further propagation of the bacteria. Cells were harvested from the remaining 4.5 ml of culture by centrifugation at 8000 x *g* for 15 min in a "Bactofuge" centrifuge. The resulting cell pellet was resuspended in 1 ml buffer D (10 mM Tris-HCl pH 8.0, 0.5 M EDTA, 10 mM NaCl) and transferred to 1.5 ml Eppendorf tubes and centrifuged for 2 min in an Eppendorf centrifuge. The supernatant was carefully removed by aspiration and the bacterial pellet was resuspended in 100 µl of ice-cold buffer A (0.1 M glucose, 20 mM CDTA, 25 mM

Tris-HCl pH 8.0) and left at room temperature for 5 min. Buffer B (0.2 M NaOH, 1 % (w/v) SDS, 200  $\mu$ l thereof) was then added, carefully mixed and placed on ice for 5 min. 150  $\mu$ l of 3 M potassium acetate, pH 4.8 was added and vortexed gently and samples were left on ice for a further 5 min. All samples were centrifuged in an Eppendorf centrifuge for 10 min. The supernatant was transferred to a new tube, 5  $\mu$ l of RNase (2 mg/ml) was added and samples were incubated at 37 °C for 30 min. An equal volume of phenol/chloroform (50 % (v/v) phenol, equilibrated with 50 mM Tris-HCl pH 8, 46 % (v/v) chloroform, 4 % (v/v) isoamylalcohol) was added, mixed by shaking and centrifuged for 5 min. The aqueous phase was removed and 2.5 volumes of cold ethanol was added to it, samples were mixed and left at -80 °C for 15 min. Samples were centrifuged for 15 min in an Eppendorf centrifuge and the resulting DNA pellet was washed with cold 70 % ethanol and vacuum dried. DNA was resuspended in 16  $\mu$ l H<sub>2</sub>O and 4  $\mu$ l of 4 M NaCl and 20  $\mu$ l of 13 % (w/v) polyethylene glycol (PEG) was added. After mixing samples were incubated at 0 °C for 30 min. The precipitated plasmid DNA was recovered by centrifugation and then washed once with cold 70 % ethanol, dried and resuspended in 20  $\mu$ l buffer D. Plasmid DNA was routinely stored at 4 °C.

### ***3.6.2 Large scale isolation of plasmid DNA for preparative purposes***

LB-medium (800 ml) supplemented with the appropriate antibiotic was inoculated with 100  $\mu$ l of a glycerol stock culture (3.6.1) of bacteria carrying the required plasmid. The culture was shaken vigorously at 37 °C and grown overnight. The bacteria were harvested by centrifugation at 8000 x g for 10 min (Beckman JA-10 rotor) and were resuspended in 10 ml of 50 mM Tris-HCl, pH 8. Then 10 ml of 0.4 % (w/v) lysozyme, 20 mM CDTA pH 8.0, 200 mM glucose was added, mixed and incubated at 4 °C for 30 min. 40 ml of 0.2 M NaOH, 1 % (w/v) SDS were added, mixed once again and left on ice for a further 10 min. The cell DNA and bacterial debris were removed by centrifugation by 8000 x g for 15 min at 4 °C (Beckman JA-10 rotor). The supernatant was filtered through Kleenex paper tissue (Kimiwipes) and the plasmid was precipitated for 30 min at room temperature by the addition of 60 ml isopropanol. Following centrifugation (8000 x g for 10 min) the supernatant was decanted and the centrifugation beaker was dried with tissue to remove excess liquid. The pellet was resuspended in 8 ml buffer D' (200 mM Tris-HCl pH 9.0, 0.5 mM EDTA, 10 mM NaCl) and transferred to

30 ml corex tubes containing 9 g caesium chloride. The contents of the tube were mixed by inversion until all the  $\text{CsCl}_2$  was dissolved, then 1 ml 0.5 % (w/v) ethidium bromide was added and mixed. The tubes were left in the dark for 30 min and then centrifuged at  $15,000 \times g$  for 25 min (Beckman JA-20 rotor). The supernatant was carefully removed and filtered through Kleenex tissue paper (Kimiwipes), transferred to ultracentrifuge tubes (Beckman Quick Seal, 16 x 76 mm) and were centrifuged at  $226,000 \times g$  (Beckman, Ti-50 rotor) for approximately 40 h at  $18^\circ\text{C}$ . Two bands of DNA were visible under U.V. light. The lower band consisting of closed circular DNA was removed (in 1 ml volume) using a needle and syringe and 2 ml of buffer D plus 8 ml ethanol was added, mixed and left overnight at  $-20^\circ\text{C}$ . The tubes were warmed to room temperature to resolubilize the precipitated  $\text{CsCl}_2$  and then centrifuged at 3,500 rpm in the Bactifuge for 40 min. The supernatant was removed and pellet vacuum dried. The pellet was resuspended in 500  $\mu\text{l}$  buffer D and transferred to Eppendorf caps. Samples were extracted once with phenol/chloroform (3.2) and twice with chloroform/isoamylalcohol (96 % (v/v) chloroform, 4 % (v/v) isoamylalcohol). DNA in the resulting aqueous phase was precipitated by the addition of 20  $\mu\text{l}$  4 M NaCl and 1 ml ethanol and pelleted by centrifugation. The DNA pellet was washed twice with cold 70 % ethanol and vacuum dried. Finally the DNA was resuspended in 100  $\mu\text{l}$  buffer D and the DNA concentration was evaluated by spectrophotometric measurement at 260 nm. Plasmid DNA was stored at  $4^\circ\text{C}$ .

### 3.7 Gel electrophoresis of DNA

Agarose gel electrophoresis was used routinely to separate, identify and purify DNA fragments larger than 200 base pairs (bp) and whose difference was greater than 50 bp. For more routine characterizing and sizing of DNA inserts resulting from restriction digests of "mini" plasmid preparations (3.6.1), polyacrylamide electrophoresis was used.

#### 3.7.1 Agarose gel electrophoresis

Agarose, 0.8 % and 1.5 % (w/v) in TAE buffer (40 mM Tris/acetate pH 7.5, 20 mM Na-acetate, 1 mM EDTA) was dissolved in a microwave oven and stored at  $65^\circ\text{C}$  for up to two weeks. Before use, the solution was cooled to below  $50^\circ\text{C}$  and ethidium bromide

was added to a final concentration of 0.5 µg/ml. Horizontal flat gels were poured and allowed to set. Gels were run in a horizontal gel trough and the electrophoresis buffer used was 1 x TAE containing 0.5 % (w/v) ethidium bromide. Samples to be run on the gel were mixed with 1/5 volume of 5 x loading buffer (15 % (w/v) Ficoll 400, 50 mM EDTA, 1 x TAE buffer, 0.5 % bromophenol blue, 0.5 % (w/v) xylene cyanol, and 0.5 % (w/v) SDS) and cooked prior to loading at 65 °C for 5 min. Routinely pBR322 plasmid, previously digested with *AluI*, was also loaded (~2 µg) as a molecular weight marker. The gels were run at 100 mV at room temperature and progress was monitored with a hand U.V. light.

### 3.7.2 Polyacrylamide gel electrophoresis

Polyacrylamide gels (6 %) were routinely used and were prepared as follows: 13 ml acrylamide solution (30 % (w/v) acrylamide, 0.8 % (w/v) bisacrylamide) 1 ml 50 x TAE, 65 µl of 40 % (w/v) ammonium persulphate, 65 µl TEMED and H<sub>2</sub>O, to a final volume of 50 ml. Vertical gels (18 cm x 15 cm x 1.5 mm) were poured. The gels were mounted in a water-cooled LKB electrophoresis apparatus. Samples to be loaded were adjusted to 1 x loading buffer using a 5 x concentrated solution (3.7.1) and incubated for 5 min at 65 °C. Gels were initially run (1 x TAE, running buffer) for up to 30 min at 100 mV, but voltage was then increased to 400 mV for the remainder of the run. Acrylamide gels were stained for 20 min in 1 x TAE containing ethidium bromide (0.5 µg/ml) and the DNA fragments were visualized by placing the gel on a U.V. transilluminator, where it could be also photographed.

### 3.7.3 DNA sequencing gels

Urea-containing polyacrylamide gels were used for analysing DNA sequencing reactions. Urea (50 g) was dissolved in 10 ml TBE (1 M Tris/borate pH 7.5, 20 mM EDTA) and 15 ml acrylamide (38 % (w/v) acrylamide, 2 % (w/v) bisacrylamide) and H<sub>2</sub>O (in an end volume of 100ml) in a water bath at approximately 40 °C. The solution was then filtered and degased, 50 µl of 40 % (w/v) ammonium persulphate and 100 µl TEMED was added, stirred gently and poured between two glass plates (40 cm x 20 cm x 0.4 mm). Prior to use, the glass plates had undergone a stringent cleaning procedure. Plates were initially treated by soaking them for 30 min in 1 M NaOH, followed by 15 min in 1 M HCl,

followed by a thorough washing with water. The plates were then washed with detergent and rinsed in deionized water and ethanol and allowed to dry. One of the glass plates was then treated with silicon (2 x 7.5 ml) and then with water and was finally polished with Kimi wipes. The gels were allowed to polymerize overnight before use. The gels were prewarmed by running them for 1 h (40 W) (electrophoresis buffer 1 x TBE) and the wells were thoroughly cleaned prior to loading with 1-2  $\mu$ l of the sequence reaction (3.13). Samples to be loaded were preincubated at 95 °C for 3 min and rapidly cooled in ice-water. The running time of the gels varied depending on which region of the sequence reaction to be read. After electrophoresis, the gel was submerged in 5 % (v/v) acetic acid until the gel was freed from the glass plate. The gel was transferred onto Whatman 3 MM paper and was dried under vacuum at 85 °C. The gels were autoradiographed (Kodak Diagnostic Film X-Omat XR) for 12-48 h, thus allowing visualization of the sequence.

### 3.8 Analysis of genomic DNA by Southern blotting

Southern blot analysis of genomic DNA isolated from both the wild-type and mutant (*cycl-1*) *N. crassa* was carried out as previously described (Southern, 1975; Maniatis *et al.*, 1982).

Genomic DNA was initially digested with various restriction enzymes and DNA fragments were resolved on a 1 % (w/v) agarose gel (3.7.1). Following electrophoresis the gel was initially soaked for 1 h in 1.5 M NaCl and 0.5 M NaOH and then for a further hour in 1 M Tris-HCl pH 8.0 and 1.5 M NaCl. The denatured DNA was transferred from the gel overnight to Biotodyne A membranes (Pall filters, Maniatis *et al.*, 1982). Filters were treated overnight at 65 °C with hybridization buffer (6 x SSC, 0.5% (w/v) SDS, 5 x Denhardt's solution, and 100  $\mu$ g/ml denatured salmon sperm DNA). The hybridization probe used was a cDNA insert (A5) containing the complete coding region for the wild-type cytochrome *c*. Hybridization to the filter-bound DNA was carried out at 65 °C in hybridization buffer containing 0.01 M EDTA and the [<sup>32</sup>P]-labelled denatured probe for 12-30 h. Filters were washed twice for 10 min in 2 x SSC, 0.5 % (w/v) SDS and twice in 2 x SSC, 0.1 % (w/v) SDS at 25 °C and finally twice for 10 min in 0.1 x SSC, 0.5 %

(w/v) SDS at 65 °C. Filters were dried at room temperature on Whatman 3MM paper and autoradiographed.

### 3.9 Analysis of RNA by Northern blotting

Poly(A)<sup>+</sup> mRNA from wild-type (74A) and mutant (*cycl-1*) *N. crassa* were resolved on 2.5 % (w/v) agarose gels containing 1.4 M formaldehyde in 0.01 M Na<sub>2</sub>HPO<sub>4</sub> pH 7.0 (Maniatis *et al.*, 1982). The samples for electrophoresis were prepared by mixing RNA (up to 10 µg) in sample buffer containing 2.2 M formaldehyde, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 % formamide, 1 % bromophenol blue and 0.4 % xylene cyanol, and incubating them at 65 °C for 5 min. Following electrophoresis the RNA was transferred overnight to Biodyne A filters (Maniatis *et al.*, 1982). The probe used in the Northern blot analysis was a nick-translated A5 cytochrome *c* cDNA insert. Prior to hybridization, the filters were prehybridized at 42 °C in hybridization buffer (3.8). Hybridization was carried out overnight at 42 °C in fresh hybridization buffer containing the [<sup>32</sup>P]-labelled denatured probe. Filters were washed four times for 5 min in 2 x SSC, 0.1 % (w/v) SDS at room temperature and twice for 15 min at 42 °C in 1 x SSC, 0.1 % (w/v) SDS. Filters were dried on Whatman 3MM at room temperature and finally, autoradiographed.

### 3.10 Purification of DNA from agarose and polyacrylamide gels

#### 3.10.1 Isolation of DNA from agarose gels

DNA inserts or linearized vectors to be purified were initially separated from other contaminating DNA species by agarose gel electrophoresis. The desired DNA fragment was identified under the U.V. lamp (366 nm) and a slice of agarose containing the band was cut out and placed in a "Biotrap" electroelution apparatus (Schleicher & Schüll) and covered with 0.5 x TAE buffer. The DNA was electroeluted at 100 mV for 30-60 min (depending on the size of the fragment) into a chamber containing 300 µl buffer. The DNA was then recovered following ethanol precipitation, the yield of DNA was usually in the range of 90 %.

### 3.10.2 Purification of oligonucleotides

Synthetic oligonucleotides used in DNA sequencing analysis (3.13) and in *in vitro* mutagenesis (3.14) were synthesized by Dr. R. Mertz, Genzentrum, München and were routinely purified prior to use. The oligonucleotides were loaded onto a urea-polyacrylamide gel (40 cm x 20 cm x 1.5 mm) which was prepared as follows: urea (100 g) was dissolved in a solution (end volume 200 ml) containing 55 ml polyacrylamide (57 % (w/v) acrylamide, 3 % (w/v) bisacrylamide) and 20 ml of 10 x TBE. The solution was filtered and degased and mixed carefully with 100 µl 40 % (w/v) ammonium persulphate and 200 µl TEMED and poured between two glass plates. The glass plates had been pretreated before as described for the sequencing gels (3.7.3). The gel was allowed to polymerize overnight.

Oligonucleotides were dissolved in 50 µl water at a concentration of approximately (5 µg/µl) by shaking for 15 min at room temperature. Formamide dye mix (100 µl, 3.9) was added and samples were treated for 5 min at 95 °C and cooled rapidly to 0 °C in ice-water. The sample (30 µl thereof) was loaded onto the gel after it had been prewarmed for 1 h (3.13). The electrophoresis was performed for 2-3 h until the bromophenol blue dye was  $\frac{2}{3}$  of the way through the gel. Using a U.V. transilluminator (356 nm) the completed oligonucleotides were visualized and the appropriate polyacrylamide slice was cut out of the gel and transferred to an Eppendorf cap. Using a sealed Eppendorf "blue pipette tip" the polyacrylamide slice was pulverized, 500 µl water was added and shaken overnight at room temperature. The sample was centrifuged (Eppendorf centrifuge, 20 min) and the supernatant was passed over a silonized pasteur pipette containing glass wool, in order to remove remaining polyacrylamide fragments. The acrylamide pellet was re-extracted with 500 µl H<sub>2</sub>O, and supernatant was also passed over the glass wool. The glass wool was washed with a further 500 µl water, the fractions were pooled and concentrated to a volume of approximately 400 µl in a "Speed-Vac". Samples were centrifuged once again (Eppendorf, 10 min) and the supernatant removed. The oligonucleotides were precipitated overnight at -80 °C in the presence of ethanol and 0.3 M Na-acetate pH 6 and 0.01M MgCl<sub>2</sub>. The oligonucleotides were recovered by centrifugation (Eppendorf, 45 min), dried and resuspended in 90 µl H<sub>2</sub>O. They were

precipitated once again as described above. Finally purified oligonucleotides were resuspended in 30  $\mu$ l and their concentration was determined spectrophotometrically.

### 3.11 Transformation of *E. coli* with plasmid DNA (Hanahan, 1983)

SOB medium (2 % (w/v) Bacto-tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM  $MgCl_2$ , 10 mM  $MgSO_4$ ), 100 ml, was inoculated with 1 ml of a stationary phase culture of *E. coli* DH1 or HB101. The culture was grown with vigorous shaking at 37 °C for 2-3 h until the O.D.<sub>550</sub> reached ~0.6. The cells were placed at 0 °C for 15 min and then centrifuged at 4 °C, 10 min at 1,500 x g (Beckman JA-20 rotor). The supernatant was discarded and cells were carefully resuspended in 30 ml TFB (10 mM MES/KOH pH 6.2, 100 mM  $RbCl_2$ , 45 mM  $MnCl_2$ , 10 mM  $CaCl_2$ , 3 mM hexamine cobalt chloride and left on ice for 15 min. Following centrifugation, the bacteria were resuspended in 2 ml TFB and placed on ice for 15 min after which 70  $\mu$ l DMSO was added, shaken and left on ice for a further 5 min. Then 70  $\mu$ l DTT (2.25 M) was added, incubated for 10 min and finally a further aliquot of DMSO (70  $\mu$ l) was added and mixed. After 5 min the cells were divided into aliquots of 200  $\mu$ l and 10  $\mu$ l DNA in ligation buffer (3.12.6) was added and incubated for 30 min at 0 °C. Samples were transferred to a water bath (42 °C) for 90 sec and then replaced immediately onto ice for 2 min. SOC medium (SOB medium supplemented with 0.4 % glucose, 800  $\mu$ l thereof), was added and cells were shaken gently (100 rpm) at 37 °C for 1 h. Routinely 100  $\mu$ l of this transformation sample was plated out onto LB-agar plates (LB medium, 2% (w/v) agar) containing the appropriate antibiotic (100 mg/l ampicillin, or 5 mg/l tetracycline or 25 mg/l chloramphenicol. The agar plates were inverted and incubated at 37 °C overnight. Single cell colonies were picked and plasmid DNA was extracted as described in section (3.6.1).

### 3.12 Enzymes used in cloning manipulations

#### 3.12.1 Digestion of DNA with restriction enzymes (Maniatis et al., 1982)

For analytical purposes (i.e. screening of "mini-prep" DNA, 3.6.1) usually 2-5  $\mu$ g plasmid DNA was digested with up to 5 units of a specific restriction endonuclease

overnight at 37 °C in a reaction volume of 20 µl. For preparative purposes (i.e. DNA insert purification or vector isolation) usually ~30 µg was taken and digested in a volume of 100 µl using the appropriate restriction enzyme at a concentration of 2 units/µg DNA. Three 10 x stock buffers with varying salt concentrations were prepared and used routinely in restriction digests for all enzymes with the exception of *SmaI* whose 10 x buffer was as follows: 20 mM KCl, 50 mM Tris-HCl pH 8.0, 10 mM MgCl<sub>2</sub>.

#### Buffers for restriction endonuclease digestion

| Buffer | NaCl   | Tris-HCl pH 7.5 | MgCl <sub>2</sub> | DTT  |
|--------|--------|-----------------|-------------------|------|
| Low    | 0      | 10 mM           | 10 mM             | 1 mM |
| Medium | 50 mM  | 10 mM           | 10 mM             | 1 mM |
| High   | 100 mM | 50 mM           | 10 mM             | 1 mM |

Following digestion each reaction was stopped by the addition of 1/5 volume of 5 x loading buffer (3.7.1).

#### **3.12.2 S1 nuclease digestion**

S1 nuclease was used to degrade the single-stranded tails from DNA fragments (generated from restriction enzymes) to produce blunt-end DNA (Henikoff, 1984). Linearized DNA (10 µg) was resuspended in 500 µl S1 buffer (0.25 M NaCl, 33 mM K-acetate pH 4.8, 1 mM ZnSO<sub>4</sub>, 5 % glycerol) containing S1 nuclease (5 units/ µg DNA). The reaction mixture was incubated for 30 min at room temperature. To stop the reaction 60 µl of S1 stop buffer was added (0.5 M Tris-HCl pH 8.0, 0.125 M EDTA) and mixed. The sample was extracted once with phenol/chloroform and once with chloroform/isoamylalcohol and finally the DNA was precipitated by adding ethanol and incubating at -80 °C for 1 h. DNA was recovered by centrifugation and washed twice with 70 % cold ethanol.

#### **3.12.3 Bal31 nuclease digestion followed by Klenow treatment**

In order to remove the 5' non-coding region of the cytochrome *c* cDNA insert, Bal31 nuclease digestion was performed as follows: the isolated insert (5 µg DNA) was dissolved in 20 µl H<sub>2</sub>O, mixed with 25 µl of 2 x Bal31 buffer (24 mM CaCl<sub>2</sub>, 24 mM

MgCl<sub>2</sub>, 0.4 M NaCl, 40 mM Tris-HCl pH 8.0, 2 mM EDTA) and was prewarmed at 25 °C for 2 min. Bal31 nuclease (2.5 units) (Lau & Gray, 1979), which had also been prewarmed (25 °C) was added and mixed. At time points corresponding to 30 sec and 45 sec, 25 µl of the reaction mixture was removed and transferred immediately to an Eppendorf cap on ice containing 2.75 µl of 200 mM EGTA and was mixed. dNTPs (3 µl each of dATP, dCTP, dGTP and dTTP, 100 mM each) and Klenow fragment of *E. coli* DNA polymerase I (5 units) was then added and incubated for 30 min at 30 °C. The reaction was extracted once with phenol/chloroform/isoamylalcohol, and twice with chloroform/isoamylalcohol. The resulting blunt-end DNA fragment was recovered by ethanol precipitation in the presence of Na-acetate, pH 6 and ligated into a pGEM3 vector cut with *Sma*I (3.12.6).

#### 3.12.4 Dephosphorylation of DNA

The terminal 5' phosphates were removed from DNA by treatment with calf alkaline phosphatase (CIP) (Chaconas & van de Sande, 1980). Plasmid DNA was digested to completion with restriction enzymes in order to obtain a linearized vector. The reaction mixture was then extracted with phenol/chloroform and the DNA was precipitated with ethanol. The DNA was subsequently dissolved in 5 µl 10 mM Tris-HCl pH 8.0 and mixed with 44 µl H<sub>2</sub>O and 5 µl 10 x CIP buffer (0.5 M Tris-HCl pH 9.0, 10 mM MgCl<sub>2</sub>, 1 mM ZnCl<sub>2</sub>, 10 mM spermidine and 0.5 units CIP. To dephosphorylate protruding 5' termini, the reaction was initially incubated at 37 °C for 30 min, then a second aliquot of CIP (0.5 units) was added and the incubation was continued for a further 30 min. To dephosphorylate DNA with blunt ends or with recessed 5' termini, the reaction was incubated for 15 min at 37 °C and then 15 min at 56 °C, a second aliquot of CIP (0.5 units) was added and the incubation periods were repeated. After the incubations 40 µl H<sub>2</sub>O and 10 µl 10 x STE (100 mM Tris-HCl pH 8.0, 1 M NaCl, 10 mM EDTA) and 5 µl 10 % (w/v) SDS was added and incubated for a further 15 min at 68 °C. The mixture was extracted once with phenol/chloroform and once with chloroform/isoamylalcohol. The aqueous phase received 1/5 volume 5 x stop buffer (3.7.1), treated for 5 min at 65 °C and was applied to a preparative agarose gel. The linearized, dephosphorylated vector was subsequently purified by gel electrophoresis followed by electroelution (3.10.1).

### 3.12.5 Phosphorylation of oligonucleotides

Single-stranded oligonucleotides (400 pmol) were incubated with T4 polynucleotide kinase (10 units) (Richardson, 1971) in a buffer containing 70 mM Tris-HCl pH 7.6, 10 mM MgCl<sub>2</sub>, 0.1 mM KCl, 5 mM DTT and 1 mM ATP, for 2 h at 37 °C in an end volume of 50 µl. The reaction was stopped by the addition of 2.5 µl EDTA (0.5 M).

### 3.12.6 Ligation of DNA

*A. with cohesive ends:* ligation of inserts with cohesive ends into a cip-treated vector with compatible cohesive ends was performed as follows, the reaction mixture contained ~500 ng DNA (molar ratio of vector:insert = 1:5) in a final volume of 20 µl of 50 mM Tris-HCl pH 7.4, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 1 mM spermidine, 1 mM ATP, 0.1 mg/ml BSA in the presence of 5 units of T4 DNA-ligase. Ligation was performed overnight at 4 °C.

*B. blunt-end ligation:* the ligation of blunt-end inserts into blunt-end cip-treated vectors was carried out in a mixture of 500 ng DNA (molar ratio of vector: insert = 1:5). The ligation reaction was in a final volume of the following buffer 25 mM Tris-HCl pH 7.4, 5 mM MgCl<sub>2</sub>, 5 mM DTT, 0.25 mM spermidine, 1 mM ATP, 1.25 mM hexamine cobalt chloride, 0.1mg/ml BSA, 13 % (w/v) PEG 6000, and in the presence of 5 units of T4 DNA ligase. Incubation was performed overnight at 4 °C followed by 4 h at room temperature.

### 3.12.7 Nick-translation of DNA (Rigby et al., 1977; Maniatis et al., 1982)

DNA which was used as a probe in colony-hybridization, Southern and Northern blots was nick-translated with [ $\alpha$ -<sup>32</sup>P]dATP. The nick-translation reaction was set up as follows: 4 µl 5 x buffer, 2.5 µl DNA fragment (0.5 -1 µg), 5 µl [ $\alpha$ -<sup>32</sup>P]dATP, 5 µl [ $\alpha$ -<sup>32</sup>P]dCTP, 1 µl *E. coli* DNA polymerase I (5 units) and was incubated for 2 h at 15 °C.

*5 x buffer* (freshly made)

50 µl 10 x buffer  
10 µl 10 mM dGTP  
10 µl 10 mM dTTP  
30 µl H<sub>2</sub>O

*10 x buffer* (can be stored at -20 °C)

500 mM Tris-HCl pH 7.2  
100 mM MgSO<sub>4</sub>  
1 mM DTT  
500 µg/ml BSA (B.R.L.)

The reaction was stopped by the addition of 25  $\mu$ l buffer D and 5  $\mu$ l 1 % (w/v) SDS, 100 mM EDTA pH 8.0 and was incubated at 65 °C for 10 min. The nick-translated DNA was separated from the free unincorporated dNTPs by chromatography on a pasteur pipette column of Sephadex G50, pre-equilibrated in buffer D. Before use in a hybridization reaction, the nick-translated DNA was denatured by cooking at 95 °C for 5 min followed by rapid cooling in ice-water.

### 3.13 Sequencing of cDNA

cDNA inserts to be sequenced were cloned into the polylinker of the plasmid pUC19 (Vierira & Messing, 1982). Sequence analysis was carried out on denatured plasmids (Chen & Seeburg, 1985) according to the dideoxy-termination method (Sanger *et al.*, 1977) using [ $^{35}$ S]-labelled dATP (Amersham) (Biggin *et al.*, 1983).

Plasmid DNA (10  $\mu$ l DNA, ~2  $\mu$ g, from "mini preps", 3.6.1) was ethanol precipitated and denatured by resuspending it in 20  $\mu$ l 0.2 M NaOH, 0.2 M EDTA and incubated for 5 min at room temperature. The mixture was neutralized by the addition of 2  $\mu$ l 2 M ammonium acetate pH 4.8 and was ethanol precipitated overnight at -20 °C. The DNA was recovered and dissolved in 10  $\mu$ l H<sub>2</sub>O and the following solutions were added: 1  $\mu$ l primer (5 pmol/ $\mu$ l), 1.5  $\mu$ l 10 x sequence buffer (50 mM Tris-HCl pH 7.5, 50 mM MgCl<sub>2</sub>, 1 mM EDTA, 50 mM DTT) and 2  $\mu$ l of [ $^{35}$ S]dATP and mixed. The samples were preincubated for 15 min at 37 °C. Then 1  $\mu$ l of Klenow fragment of *E. coli* DNA polymerase (2 unit/ $\mu$ l) was added and then 2  $\mu$ l of the reaction mixture was added to four caps containing 3  $\mu$ l of a nucleotide mix containing either ddATP, ddCTP, ddGTP or ddTTP.

| <u>"A-reaction"</u> |             | <u>"C-reaction"</u> | <u>"G-reaction"</u> | <u>"T-reaction"</u> |
|---------------------|-------------|---------------------|---------------------|---------------------|
| dGTP                | 100 $\mu$ M | 100 $\mu$ M         | 5 $\mu$ M           | 100 $\mu$ M         |
| dCTP                | 100 $\mu$ M | 10 $\mu$ M          | 100 $\mu$ M         | 100 $\mu$ M         |
| dTTP                | 100 $\mu$ M | 100 $\mu$ M         | 100 $\mu$ M         | 5 $\mu$ M           |
| ddATP               | 125 $\mu$ M | ddCTP 100 $\mu$ M   | ddGTP 300 $\mu$ M   | ddTTP 1200 $\mu$ M  |

Nucleotide solutions were made in 1 x sequence buffer.

Samples were incubated for 20 min at 30 °C, then 1 µl "chase" solution was added (0.125 mM from all four dNTPs) and incubated for a further 15 min at 30 °C. Samples were dried in a "Speed-Vac" and were resuspended in 4 µl of formamide-dye mix (0.1% (w/v) xylene cyanol, 0.1 % (w/v) bromophenol blue, 10 mM EDTA, 95 % (v/v) deionized formamide). Samples were cooked at 95 °C for 3 min and rapidly cooled in ice-water prior to their loading on sequence gels.

### 3.14 Oligonucleotide site-directed mutagenesis by the gapped-duplex method

For the construction of the plasmids encoding fusion proteins pSc<sub>1</sub>-c and pLc<sub>1</sub>-c, oligonucleotide site-directed mutagenesis was performed to introduce unique *Pvu*II sites in the cytochrome *c*<sub>1</sub> cDNA sequence. The *Eco*RI/*Hind*III fragment of *N. crassa* cytochrome *c*<sub>1</sub>, which encodes the 5' non-coding region and the amino-terminal half of the cytochrome *c*<sub>1</sub> protein (Römisch *et al.*, 1987) was cloned into the plasmid pMa5-8 (Fritz *et al.*, 1986).

#### 3.14.1 Preparation of single-stranded DNA.

To prepare single-stranded plasmid DNA (ssDNA), the above mentioned recombinant pMa5-8 plasmid was cloned in *E. coli* WK6. An overnight culture of these cells (grown in LB medium supplemented with 100 µg/ml ampicillin) was diluted 1:50 in LB medium without antibiotic and was incubated at 37 °C with constant shaking (160 rpm). When the O.D.<sub>650</sub> reached 0.1, the culture was infected with a helper phage (M13K07) at a m.o.i. of ~20, and the culture was incubated for a further 4-5 h. A portion of the culture (25 ml) was removed and centrifuged (Beckman JA-20 rotor, 17,400 x g for 10 min) to remove the cells. The supernatant (20 ml thereof) was mixed with 5ml PEG solution (20 % (w/v) polyethylene glycol 6000, 2.5 M NaCl) and left at room temperature for 15 min. The phages were recovered by centrifugation (Beckmann JA-20 rotor, 11,000 x g for 10 min) and were resuspended in 500 µl TE buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA) and extracted with an equal volume of TE-saturated phenol. The aqueous phase was re-extracted with chloroform/isoamylalcohol and the liberated ssDNA was precipitated by adding 1/10 the volume of 5 M NH<sub>4</sub>Cl and one volume of isopropanol and was collected

by centrifugation (Eppendorf, 20 min). The ssDNA was dissolved in 50  $\mu$ l TE-buffer. The yield of ssDNA was typically 1.5-5  $\mu$ g/ml culture.

### 3.14.2 Formation of the "gapped-duplex" DNA

The plasmid pMc5-8 was cut with *EcoRI/HindIII* and the linearized plasmid was purified following agarose gel electrophoresis and electroelution. The vector (0.1 pmol) was mixed with 0.5 pmol ssDNA, 5  $\mu$ l of 1.5 M KCl, 100 mM Tris-HCl pH 7.5, in an end volume of 40  $\mu$ l. The reaction mixture was heated for 5 min at 95 °C and then for 20 min at 65 °C. An aliquot (20  $\mu$ l) was electrophoresed on an agarose gel to check the formation of the gapped-duplex DNA (gdDNA).

### 3.14.3 Hybridization of the mutagenic oligonucleotide to the gdDNA and the fill-in reaction.

Two purified oligonucleotides, d(5'GACTCGGCAGCTGCGCCGGAGCTC3') and d(5'CCTTCCTCAGCTGGAGTCATGGCGG3') were employed. Annealing of the mutagenic oligonucleotides to the gdDNA was achieved as follows: The gdDNA (8  $\mu$ l) was mixed with 2  $\mu$ l phosphorylated oligonucleotides (20 pmol) and incubated for 5 min at 65 °C and then cooled slowly to room temperature.

H<sub>2</sub>O (24  $\mu$ l) and 4  $\mu$ l 10 x fill-in buffer (625 mM KCl, 275 mM Tris-HCl pH 7.5, 150 mM MgCl<sub>2</sub>, 20 mM DTT, 0.5 mM ATP and 0.25 mM of the four dNTPs) were added along with 1  $\mu$ l Klenow fragment of the *E. coli* DNA polymerase I (1 unit/ $\mu$ l). The sample was incubated for 45 min at room temperature (Levinson *et al.*, 1984).

### 3.14.4 Transformation into *E. coli* WK6 cells

An aliquot (5  $\mu$ l) of the filled-in gdDNA was used to transform competent *E. coli* WK6*mutS* cells (3.11). Transformants were selected for by resistance to chloramphenicol (25 mg/l), resistance to chloramphenicol being encoded for by the gapped strand and hence contains the mutated oligonucleotide. Positive transformants, i.e. those containing the introduced unique *PvuII* site in the cDNA insert, were selected and plasmid DNA was prepared (3.6.1) and used to transform competent *E. coli* WK6 cells.

### 3.15 Synthesis of mitochondrial precursor proteins *in vitro*

Full-length cDNA inserts coding for cytochrome *c*, cytochrome *c*<sub>1</sub> (Rörmisch *et al.*, 1987; Hartl *et al.*, 1987)  $\beta$ -subunit of F<sub>1</sub>-ATPase (Pfaller *et al.*, 1989) and porin (Kleene *et al.*, 1987) were isolated from the *N. crassa* library. All the cDNA inserts were cloned into pGEM3,4 plasmids and could be transcribed with SP6 RNA polymerase. Initially, however, wild-type and mutant (*cyc1-1*) cytochrome *c* inserts were cloned in the pDS5 vector (Stueber *et al.*, 1984). However, they were not very efficiently transcribed by the *E. coli* RNA polymerase owing to the presence of GC-tails introduced during the cloning procedure (Galili *et al.*, 1986). The 5' noncoding region was shortened by ~80 bp with Bal31 (3.12.3) and ligated into the *Sma*I site of pGEM3 plasmid. *E. coli* strain DH1 were transformed (3.11) and clones oriented in the SP6 direction were selected.

#### 3.15.1 Transcription of cDNA inserts cloned in pDS5 by *E. coli* RNA polymerase

Plasmid DNA (~40  $\mu$ g) was dissolved in water and 24  $\mu$ l Mix I (150 mM Hepes/KOH, pH 7.5, 75 mM Mg-acetate, 1.5 M K-acetate, 15 mM spermidine, 37.5 mM DTT), 10  $\mu$ l 10 mM NTP-ATP, 10  $\mu$ l RNasin (1 unit/ $\mu$ l), 20  $\mu$ l 7mGpppA, 24  $\mu$ l H<sub>2</sub>O and 2  $\mu$ l *E. coli* RNA polymerase (6 units/ $\mu$ l) were added. Following a 5 min incubation at 37 °C, 20  $\mu$ l 10 mM ATP was added and the incubation was continued for a further 15 min. DNA and synthesized RNA were precipitated by the addition of ethanol and recovered by centrifugation. Following washing with 70 % cold ethanol, the pellet was resuspended in 70  $\mu$ l H<sub>2</sub>O and added to 180  $\mu$ l of nuclease-treated reticulocyte lysate (3.15.3).

#### 3.15.2 Transcription of cDNA inserts cloned in pGEM3,4 by SP6 RNA polymerase

Plasmids having an SP6 promoter (Melton *et al.*, 1984) located before the cDNA encoding precursor proteins were transcribed with SP6 RNA polymerase. In a final volume of 100  $\mu$ l the following were mixed together: 60  $\mu$ l Pre-mix A, 2  $\mu$ l RNasin (40 units/ $\mu$ l), 5  $\mu$ l 7mG(5')ppp(5')G (5 mM), pGEM plasmid (6  $\mu$ g) and SP6 RNA polymerase (40 units).

Pre-mix A: 1 ml 10 x salt mix, 20 µl BSA (50 mg/ml), 200 µl DTT (0.5 M),  
50 µl each of ATP, CTP and UTP (0.1M), 5 µl of GTP (0.1 M).

*10 x salt mix* : 60 mM Mg-acetate, 20 mM spermidine,  
400 mM Hepes/KOH, pH 7.4.

The transcription reaction was carried out at 40 °C for 45 min. DNA plus RNA was precipitated following the addition of  $1/10$  volume of 10 M LiCl and 3 volumes of ethanol (Merck p.A) and stored overnight at -80 °C. The RNA was recovered after centrifugation (Beckman, JA-20 20 000 rpm, 20 min) and dissolved in 100 µl H<sub>2</sub>O and was added to 400 µl reticulocyte lysate for translation (3.15.3).

### 3.15.3 *In vitro* translation in a rabbit reticulocyte lysate system

The reticulocyte lysates were prepared stored at -80 °C in aliquots of 0.4 ml (Hunt & Jackson, 1974). *In vitro* protein synthesis was performed using a modified procedure of Pelham & Jackson, 1976. One portion of lysate (0.4 ml) was thawed slowly in the presence of 12.5 µl hemin solution (1 mM hemin in 90 % (v/v) ethylene glycol, 2 mM Tris-HCl, 5 mM KCl, pH 8.2). The lysates were first nuclease treated which involved the addition of 5 µl CaCl<sub>2</sub> (0.1 M) and 5 µl nuclease (from *Staphylococcus aureus*, 1 mg/ml) and incubation for 15 min at 20 °C. Nuclease activity was inhibited by the addition of 25 µl EGTA (0.1 M) and the reaction was placed on ice. To 220 µl of nuclease-treated lysate the following were added:

50 µl L-[<sup>35</sup>S]cysteine (1100-1400 Ci/mmol, Amersham)  
or 35 µl L-[<sup>35</sup>S]methionine (1000 Ci/mmol, Amersham)  
20 µl amino acid mix, 0.5-3 mM of all L-amino acids with exception of either  
cysteine or methionine, depending on which [<sup>35</sup>S]amino acid used,  
20 µl KCl (2 M),  
20 units RNasin,  
20 µl creatine phosphate (0.2 M),  
5 µl creatine kinase (5 mg/ml in 50 % (v/v) glycerol),  
100 µl transcription mix,  
H<sub>2</sub>O to an end volume of 500 µl.

Translation was performed for 1 h at 37 °C, after which the lysate was placed on ice and 44 µl of either unlabelled cysteine (15 mM) or methionine (15 mM) as "chase" was

added. A post-ribosomal supernatant was prepared by centrifugation (Beckman L8-M ultracentrifuge, Ti-50 rotor, 266,000  $\times$  g, 60 min) and was made iso-osmotic (for mitochondria) by the addition of sucrose to a final concentration of 0.25 M. The lysates were portioned into aliquots of 100  $\mu$ l and stored at -80 °C under nitrogen gas.

### 3.16 Growth of *Neurospora crassa* cultures.

*Neurospora crassa*, wild-type (74A) was grown for 14 h at 25 °C with vigorous aeration in Vogel's minimal medium supplemented with 2 % (w/v) sucrose (Hennig & Neupert, 1983). The cultures were inoculated with  $1 \times 10^6$  conidia/l and grown under bright illumination. The *cyt2-1* and *cycl-1* *N. crassa* mutants were grown under identical conditions for 40-48 h. Cytochrome *c* content in the mitochondria isolated from these *N. crassa* mutants was routinely checked by difference spectrum analysis as described by Bertrand & Pittenger (1972). Additionally in the case of the *cyt2-1* mutant, deficiency in CCHL activity was also controlled enzymatically.

### 3.17 Cell disruption and isolation of mitochondria.

Hyphae (1 g, wet weight) were ground with sterile quartz sand (1.5 g) and a buffer (2 ml) containing 250 mM sucrose, 2 mM EDTA, 10 mM Mops-KOH, pH 7.2 (SEM-buffer) plus 2 mM PMSF, added from a freshly prepared stock solution in ethanol, for 1-2 min with a mortar and pestle at 0-4 °C. PMSF was omitted from SEM buffer in experiments where protease treatment was involved. Cell-free homogenate was obtained by centrifuging the resulting slurry twice for 5 min at 2,000  $\times$  g (Beckman JA-20 rotor) and the pellets were discarded. A crude mitochondrial fraction was obtained by centrifuging the resulting homogenate for 12 min at 17,400  $\times$  g. Mitochondria were then washed by resuspension in SEM and recentrifuged for a further 12 min at 17,400  $\times$  g.

### 3.18 Pretreatment of mitochondria with protease.

Freshly isolated mitochondria were resuspended in 1.0 ml of SEM buffer at a protein concentration of 1 mg/ml and cooled to 0 °C. Proteinase K was added (from a 1 mg/ml)

stock solution) to a concentration of 50 µg/ml and incubated at 0 °C for 30 min. Protease activity was then inhibited by the addition of 1.0 ml of SEM-buffer containing 2 mM PMSF. After a further 5 min incubation, the mitochondria were reisolated by centrifugation 17,400 x g for 12 min. The mitochondrial pellet was then resuspended in fresh SEM buffer at a concentration of 2 mg/ml. No protease activity was detected following this treatment (Nicholson *et al.*, 1988; results not shown).

### **3.19 Import of cytochrome *c* into mitochondria and measurement of holocytochrome formation (Nicholson *et al.*, 1987a).**

#### **3.19.1 Import of apocytochrome *c*.**

Apocytochrome *c* was synthesized in nuclease-treated rabbit reticulocyte in the presence of L-[<sup>35</sup>S]cysteine as previously described (3.15.3). Unless otherwise indicated, binding and import of apocytochrome *c* were performed as follows: mitochondria (50 µg) were incubated in a mixture (final volume 100 µl) containing BSA buffer (3 % (w/v) bovine serum albumin, 70 mM KCl, 220 mM sucrose, 10 mM Mops-KOH, pH 7.2), 3 µM hemin and 15 µl [<sup>35</sup>S]cysteine-labelled reticulocyte lysate. The reaction was started by adding NADH (5 mM final concentration) or sodium dithionite (freshly prepared to a final concentration of 1 mg/ml) (Nicholson and Neupert, 1988) and incubating at 25 °C for 15 min. Reducing agents were omitted in cases where apocytochrome *c* binding, but not holo-formation, was required. The reaction mixture was then prepared for immunoprecipitation by 1 ml of buffer containing 1 % (w/v) Triton X-100, 0.3 M NaCl and 10 mM Mops/KOH, pH 7.2.

#### **3.19.2 Measurement of holocytochrome *c* formation.**

The amount of apocytochrome *c* and synthesized holocytochrome *c* was determined essentially as described by Nicholson *et al.*, 1987a: Following the lysis of import samples with Triton X-100 containing buffer, samples were shaken at 4 °C for 30 min and then were clarified by centrifugation for 15 min at 27,200 x g (Beckman JA-20 rotor). The supernatant was then added to preswollen Protein A-Sepharose CL-4B (Pharmacia, Uppsala) prebound with antisera directed against both apo- and holocytochrome *c*. Samples were shaken at 4 °C for 1 h and the immunocomplex was reisolated by

centrifugation for 1 min in an Eppendorf centrifuge. The pellets were washed twice with 1 ml of 1 % (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris-HCl pH 7.2, once with 1 ml of 1 mM  $K_3[Fe(CN)_6]$  in 10 mM Tris-HCl pH 7.0 (to oxidize cytochrome *c* for uniform tryptic digestion) and finally twice with 1 ml 10 mM Tris-HCl pH 7.0. The immunocomplex was dissociated in 8 M urea (60  $\mu$ l) by shaking at room temperature followed by 30 min at 56 °C and then additional shaking at room temperature for 15 min. The immunocomplex was then digested with trypsin following the addition of 250  $\mu$ l of 60 mM  $NH_4CO_3$  containing 25  $\mu$ g of purified *Neurospora crassa* holocytochrome *c* (for a marker) and 50  $\mu$ g trypsin (type XIII from bovine pancreas, treated with tosylphenylalanylchloromethane (10,000 unit/mg), Sigma) for 1 h at 37 °C. The reaction was halted by the addition of 75  $\mu$ g soybean trypsin inhibitor (Type I-S, Sigma).

The resulting tryptic peptides were resolved by reverse-phase HPLC on a Waters Associates (Milford)  $\mu$ Bondapak phenyl column (Nicholson *et al.*, 1987a). The cysteine-containing tryptic peptide from apocytochrome *c* eluted at ~35 min following injection and was collected in order to quantify levels of apocytochrome *c*. For determination of cytochrome *c* heme lyase activity, the holocytochrome *c* cysteine-containing tryptic peptide was collected (by monitoring the marker holocytochrome *c* peptides at 405 nm) and the radioactivity was measured.

### 3.20 Import of cytochrome *c*<sub>1</sub>, p*Sc*<sub>1</sub>-*c* and p*Lc*<sub>1</sub>-*c* into mitochondria

The precursors of cytochrome *c*<sub>1</sub>, *Sc*<sub>1</sub>-*c* and *Lc*<sub>1</sub>-*c* were synthesized in rabbit reticulocyte lysate in the presence of L-[<sup>35</sup>S]methionine or [<sup>35</sup>S]cysteine as indicated (3.15.3). Synthesis was directed by transcripts from pGEM plasmids as previously described (3.15.2). Unless otherwise indicated, mitochondria (50  $\mu$ g) were incubated for 20 min at 25 °C in a mixture (total volume 100  $\mu$ l) containing 3 % (w/v) bovine serum albumin, 70 mM KCl, 220 mM sucrose, 2.5 mM  $MgCl_2$ , 10 mM Mops-KOH, pH 7.2 and 15  $\mu$ l of [<sup>35</sup>S]-labelled precursor reticulocyte lysate. Where indicated, the matrix-localized processing peptidase was inhibited by the addition of 5 mM EDTA and 0.2 mM *o*-phenanthroline (Schmidt *et al.*, 1984). Dissipation of the mitochondrial membrane potential ( $\Delta\Psi$ ) was achieved by the addition of a mixture of antimycin A, oligomycin and

valinomycin, (added from a 100-fold concentrated stock solution in ethanol) to final concentrations of 8  $\mu\text{M}$ , 20  $\mu\text{M}$  and 0.5  $\mu\text{M}$ , respectively. Following incubation, the import mixtures were cooled to 0 °C and treated with 20  $\mu\text{g}$  proteinase K/ml (from *Tritachium album*, Boehringer) for 30 min at 0 °C. For analysis of cytochrome  $c_1$  import, samples were diluted with SEM buffer containing 2 mM PMSF, mitochondria were reisolated by centrifugation at 17,400  $\times g$  for 12 min (Beckman JA-20 rotor), lysed with SDS-containing sample buffer and resolved by SDS/PAGE (3.24). For analysis of pSc $_1$ -c and pLc $_1$ -c import proteinase K activity was stopped by the addition of 1 ml 1 % (w/v) Triton X-100, 0.3 M NaCl, 10 mM Mops-KOH pH7.2, containing 2 mM PMSF. Samples were immunoprecipitated using an antiserum directed against cytochrome  $c$  (i.e. apo- plus holocytochrome  $c$ ). Immunoprecipitates were analysed either by HPLC to quantify apo- and holopeptides (3.19.2) or by SDS/PAGE. In all cases, radioactive bands in the dried polyacrylamide gels were visualized by fluorography and quantified by laser densitometry of the resulting film. The amount of protease-resistant cytochrome  $c_1$ , Sc $_1$ -c and Lc $_1$ -c are expressed in arbitrary units.

### 3.21 Enzymatic Tests

#### 3.21.1 Adenylate kinase

Adenylate kinase activity was assayed according to Schmidt *et al.*, 1984. The following solutions were mixed together:

- 54  $\mu\text{l}$  Mix B (0.7 ml of 20 mg/ml NADH plus 0.35 ml of 10.5 mg/ml PEP),
- 9  $\mu\text{l}$  of 55 mg/ml ATP,
- 9  $\mu\text{l}$  of 3 mg/ml oligomycin in ethanol,
- 9  $\mu\text{l}$  of 0.4 mg/ml rotenone in acetone,
- 18  $\mu\text{l}$  pyruvate kinase/lactate dehydrogenase (LDH) mix (50  $\mu\text{l}$  of 2 mg/ml pyruvate kinase plus 7  $\mu\text{l}$  of 5 mg/ml LDH plus 1 ml 50 % (v/v) glycerol in water),
- 50-100  $\mu\text{l}$  of sample (~17.5  $\mu\text{g}$  mitochondrial protein, solubilized in 1 % Genapol
- 1.8 ml of 130 mM KCl, 6 mM MgCl $_2$ , 100 mM Tris-HCl, pH 7.5.

The mixture was divided between two cuvettes (each 850  $\mu\text{l}$ ) and the reaction was started by the addition of 5  $\mu\text{l}$  of AMP (7.4 mg/ml) to the reference cuvette. Activity was

followed by measuring a decrease in the absorbance at 366 nm resulting from the oxidation of NADH to NAD<sup>+</sup>.

### 3.21.2 Fumarase

Fumarase activity was measured according to the method of Kanarek & Hill, 1964, as follows:

0.65 ml of 25 mM K-phosphate buffer pH 7.2 was mixed with the sample to be assayed (usually 10 µg mitochondrial protein, previously solubilized in 1 % (w/v) Genapol). The reaction was started by the addition of 50 µl of 50 mM malate. Enzyme activity was monitored by the increase in absorbance at 240 nm at 30 °C.

## 3.22 Immunoprecipitation

Where indicated immunoprecipitation techniques were employed to determine the relative concentrations of specified proteins in various mitochondrial fractions and of the *in vitro* synthesized proteins in reticulocyte lysate. Immunoprecipitation of all specified proteins was carried out under the following conditions. Fractions to be assayed were adjusted to 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris-HCl pH 7.5, final volume 1 ml, and were shaken for 30 min at 4 °C. Samples were subjected to a clarifying spin for 15 min at 27,000 x g (Beckman, JA-20 rotor). The supernatant was added to preswollen Protein A-Sepharose CL-4B (Pharmacia, Uppsala) prebound to specific antiserum (1 mg Protein A-Sepharose/ 10 µl serum). Appropriate amounts of antiserum preparations, as determined by titration (usually 30-50 µl) were used. Samples were shaken at 4 °C for 1 h. The immunocomplex was then reisolated by centrifugation in an Eppendorf bench centrifuge, and was then washed three times with Triton X-100 buffer (1 % (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5) and twice with a 10 mM Tris-HCl, pH 7.5 solution. The immunocomplex was dissociated by shaking for 10 min at 4 °C with 50 µl of sample buffer (2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 10 % (v/v) glycerol, 60 mM Tris-HCl, pH 6.8) and boiling for 3 min at 95 °C. After centrifugation an aliquot of the supernatant was taken and analysed by electrophoresis and fluorography.

### 3.23 SDS-polyacrylamide gel electrophoresis

Vertical gel electrophoresis was performed as according to Laemmli, 1970. The gels were formed between two glass plates (130 mm x 160 mm) according to the schemes below. For gel analysis of cytochrome *c*, pSc<sub>1</sub>-*c* and pLc<sub>1</sub>-*c* a gel whose final concentrations of acrylamide/bisacrylamide were 14 %/ 0.6 % (w/v) respectively. For gel analysis of cytochrome *c*<sub>1</sub> and F<sub>1</sub>B, a lower bisacrylamide, namely, 17.5 % acrylamide, 0.2 % (w/v) bisacrylamide, was used. Samples for electrophoresis were prepared by cooking in sample buffer (see above) for 5 min at 95 °C. Electrophoresis was performed overnight at 3 mA. The gels were then stained for 20 min with 0.2 % (w/v) Coomassie Brilliant Blue in destain solution. Gels were then washed with water and allowed to destain by shaking in the following destain solution: methanol:acetic acid:water (4:1:5, v/v/v).

#### *Lower bisacrylamide gel*

| <i>Solution</i>         | <i>Base Gel</i> | <i>Running Gel</i> | <i>Stacking Gel</i> |
|-------------------------|-----------------|--------------------|---------------------|
| 30 % acrylamide         |                 |                    |                     |
| 0.2 %bisacrylamide      | 6.7 ml          | 9.7 ml             | 0.83 ml             |
| 1.875 M Tris-HCl pH 8.8 | 2 ml            | 3.5 ml             | -                   |
| 0.6 M Tris-HCl pH 6.8   | -               | -                  | 0.5 ml              |
| H <sub>2</sub> O        | 1.1 ml          | 3.5 ml             | 3.6 ml              |
| 10 % (w/v) SDS          | 100 µl          | 167 µl             | 50 µl               |
| TEMED                   | 25 µl           | 6 µl               | 5 µl                |
| 10 % AMPS               | 50 µl           | 84 µl              | 25 µl               |

#### *Higher bisacrylamide gel*

| <i>Solution</i>         | <i>Base gel</i> | <i>Running Gel</i> | <i>Stacking gel</i> |
|-------------------------|-----------------|--------------------|---------------------|
| 30 % acrylamide         |                 |                    |                     |
| 0.6 % bisacrylamide     | 6.7 ml          | 7.9 ml             | 0.83 ml             |
| 1.875 M Tris-HCl pH 8.8 | 2.0 ml          | -                  | -                   |
| 1 M Tris-HCl pH 8.8     | -               | 6.5 ml             | -                   |
| 0.6 M Tris-HCl pH 6.8   | -               | -                  | 0.5 ml              |
| H <sub>2</sub> O        | 1.1 ml          | 2.3 ml             | 3.6 ml              |
| 10 % SDS                | 100 µl          | 167 µl             | 50 µl               |
| TEMED                   | 25 µl           | 6 µl               | 5 µl                |
| 10 % AMPS               | 50 µl           | 84 µl              | 25 µl               |

### 3.24 Fluorography

Gels were prepared for fluorography by soaking them for 30 min in Amplify (Amersham), dried under vacuum and exposed to X-ray films (Kodak, Omat X-R) at -80 °C. Bands on resulting fluorograms were then quantified by laser densitometry.

### 3.25 Determination of radioactivity

Radioactivity determinations were performed by scintillation counting in 100 µl 10 % (w/v) SDS, 100mM Tris-HCl, pH 8.0 plus 7.5 ml ACS II (Amersham). The radioactivity (cpm) was then determined in a liquid scintillation counter (Type Tri-Carb, Packard Instruments).

### 3.26 Protein estimation (Bradford, 1976)

Protein estimation of mitochondrial preparations was performed as described by Bradford, 1976. Mitochondrial suspension (50 µl) was diluted with 450 µl H<sub>2</sub>O. BSA standards and appropriately diluted standards were mixed with 2.5 ml of 1:4 diluted dye reagent:H<sub>2</sub>O (BioRad Protein Assay) and vortexed gently. Absorbance at 595 nm was measured after 5 min at room temperature. Protein concentrations of the assays were thus calculated from the standard curve obtained.

### 3.27 Miscellaneous

Published methods were used for the preparation of holocytochrome *c* and apocytochrome *c* and for the production of specific antibodies in rabbits (Hennig & Neupert, 1983). Reticulocyte lysates were prepared according to Hunt and Jackson, 1974. Isolation of porin and preparation of water-soluble porin was performed as previously described (Pfaller et al., 1985; Pfaller & Neupert, 1987) and was kindly donated by Dr. R. Pfaller.

### 3.28 Chemicals and equipment

#### 3.28.1 Chemicals

The chemicals used in this study were obtained from the following companies:

Amersham-Buchler, Braunschweig:

[ $^{35}\text{S}$ ]Cysteine (1100-1400 Ci/mmol), [ $^{35}\text{S}$ ]methionine (1000 Ci/mmol)

BioRad, München:

Protein reagents and standards for Bradford protein determination

Boehringer Mannheim:

Alkaline phosphatase (calf intestine), AMP, ATP, Bal31 exonuclease, BSA (molecular biology quality), caesium chloride, creatine kinase, DTT, ethidium bromide, Klenow fragment from *E. coli* DNA polymerase I, NADH, nuclease (from *Staphylococcus aureus*), n-octylglucoside, phosphoenolpyruvate, pyruvate kinase/LDH, proteinase K (from *Tritrachium album*), restriction endonucleases, RNase H, T7 RNA polymerase and *E. coli* RNA polymerase

BRL, Gaithersburg, MD, USA:

Agarose, Ultrapure

Difco Lab., Detroit, Mich. USA:

Bacto-peptone, nutrient broth, Bacto-agar, Bacto-tryptone and yeast extract

Dupont New England Nuclear, Boston, USA:

[ $\alpha^{35}\text{S}$ ]dATP (1500 Ci/mmol) and [ $\gamma^{32}\text{P}$ ]ATP (2000 Ci/mmol)

Hoechst, Frankfurt:

Genapol X-100

Kodak, München:

X-ray films (X-Omat XR)

Pharmacia P-L Biochemicals Freiburg:

Cap-analogue 7mG(5')ppp(5')G, Ficoll 400, NTPs, dNTPs, ddNTPs, Protein A Sepharose CL-4B, Sephadex G25, S1 nuclease T4 polynucleotide kinase and terminal deoxyribonucleotidyl transferase

Promega Biotec:

RNase-inhibitor (RNasin from human placenta), SP6 RNA polymerase

Serva, Heidelberg:

Acrylamide, ampicillin, bisacrylamide, bromophenol blue, sucrose, SDS, spermidine, TEMED, tetracycline and urea

Sigma, München:

AMPS, antimycin A, apyrase (grade VIII from potato), BSA, creatine phosphate, DNA from salmon sperm, hemin, HEPES, mercaptoethanol, MES, oligomycin, PEG 8000, PIPES, PVP, soyabean trypsin inhibitor (Type I-S), TFA, TMPD, trypsin (type XIII from bovine pancreas, treated with tosylphenylalanylchloromethane, 10,000 unit/mg) and valinomycin

All other chemicals were obtained from Merck, Darmstadt

### 3.28.2 *Equipment*

Abimed, Düsseldorf:

Gilson pipettes

Agfa Gaveart, München:

X-Ray developing machine Gevomatic 60

Beckman Instr., München:

Airfuge, Cooling centrifuge J2-21, and Ultracentrifuge (L8 series)

Bender and Hobein, Zürich/München:

Vortex mixer

Eppendorf, Hamburg:

Pipettes, shakers, heating blocks and bench centrifuges

Heraeus Christ, Osterode:

Bench centrifuges, Bactifuge and Biofuge

Ika, Staufen:

Magnetic stirrers

LKB, München:

Apparatus for DNA electrophoresis, laser densitometry Ultrascan XL

Packard Instr. Comp., Downes Grove Ill. USA:

Liquid scintillation counter TriCarb 460

Pharmacia, Freiburg:

Columns for chromatography, flow-through monitores, fraction collectors and power supplies for electrophoresis

Waters-Millipore, Millford, MA:

HPLC apparatus: pumps 510, photometer 490, WISP 710B, Automated gradient controller,  $\mu$ Bondapak phenyl column (3.9 mm x 30 cm, 10  $\mu$ m particle size)

## 4. RESULTS

The problem is posed as to how apocytochrome *c* which does not contain a removable presequence is targeted to mitochondria. Studies involving a protein from the outer membrane, which does not undergo proteolytic processing upon import, namely, the 70 kD protein from yeast mitochondria have suggested that the targeting information is also contained in its amino terminus (Riezman *et al.*, 1983a; Hase *et al.*, 1984). Does apocytochrome *c* contain such an internal amino-terminal targeting sequence also? A cytochrome *c* mutant of *Neurospora crassa* known as *cyc1-1* was thus studied. Phenotypically, this mutant has a slightly reduced growth rate when compared to that of the wild-type form, and spectral analysis has indicated that holocytochrome *c* levels are reduced to 10-20 % of wild-type level, whereas levels of other cytochrome components of the respiratory chain are unaltered (H. Bertrand, personal communication). The mutation does not affect the biogenesis of cytochrome *c* at the level of transcription or translation of the cytochrome *c* mRNA (B. Hennig & W. Neupert, unpublished data). Apocytochrome *c* is, in fact, synthesized in the cell, albeit in an altered form which is incompetent for import into mitochondria. To investigate the specific details of this alteration in the apocytochrome *c* protein, sequence studies of wild-type and mutant cytochrome *c* cDNA clones were performed. The similarities and differences of the wild-type cytochrome *c* mRNA, compared with those of the mutant, are discussed with reference to the targeting of apocytochrome *c* to mitochondria.

### 4.1 Isolation of cytochrome *c* cDNAs and genomic DNA

A cDNA library prepared from *Neurospora crassa* (74A, wild-type) poly(A)<sup>+</sup> mRNA was cloned into the *Pst*I site of pBR322 by dG/dC tailing (3.3). Recombinant clones were screened using a 280 bp apocytochrome *c* cDNA insert (A12/20), identified by hybridization selection of mRNA, as a [<sup>32</sup>P]-labelled hybridization probe (3.5.1). Of the cytochrome *c* recombinant clones that were isolated, the largest insert identified, A5, was 680 bp. Northern blot analysis, where poly(A)<sup>+</sup> mRNA and the 680 bp insert were resolved on agarose-formaldehyde gels, confirmed that the insert A5 represents a full-length cDNA for cytochrome *c* (results not shown).

A *cyc1-1* mutant cDNA library was then constructed and screened for cytochrome *c* clones using the A5 cDNA insert. The longest insert detected (610 bp) did not represent a full-length cDNA as shown by Northern analysis (results not presented). Sequence analysis confirmed, however, that the complete coding region for the cytochrome *c* was contained in this insert.

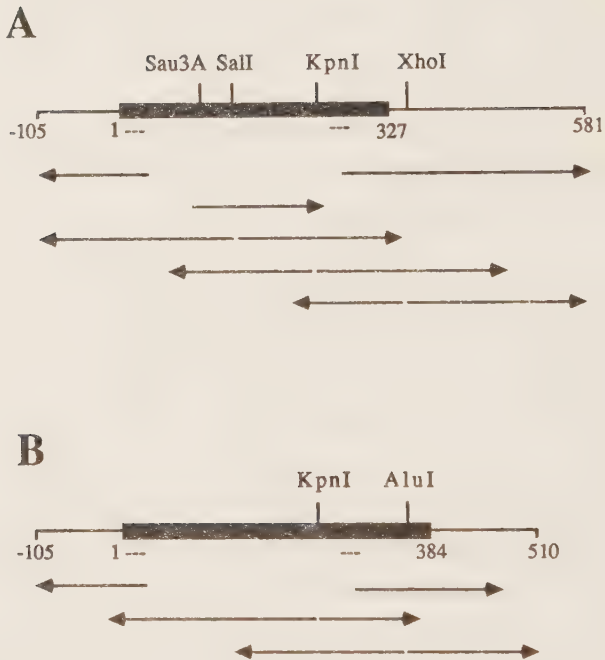
The 680 bp insert of the wild-type cDNA was also used to screen a *N. crassa* wild-type genomic library. Approximately 20,000 clones were screened and 21 positive clones were identified and isolated for further analysis.

## 4.2 Sequencing of the cytochrome *c* cDNAs and genomic DNA

Both the wild-type and mutant cytochrome *c* cDNAs were sequenced in the following manner. Plasmids containing hybridization-positive cDNA fragments were isolated (3.6.2) and digested with *Pst*I. The resulting inserts were further digested with various restriction enzymes (see Figure 1) and ligated into the vector pUC19. The sequences of all fragments of the wild-type cDNA were determined on both strands, except for the two end fragments which were not determined from the tail ends.

The 5' and 3' ends of both cytochrome *c* cDNAs were also sequenced using cytochrome *c*-specific synthetic primers. The primers 5'GGCACCCCTTCTTGAATC3' and 5'GGCCTTCGGTGGTCTCAAGAAG3' hybridized specifically to nucleotide positions corresponding to codons 6-12 (anti-sense strand) and 85-91 (sense strand) respectively, of the wild-type cytochrome *c* cDNA.

The cytochrome *c* gene was only partially sequenced. The cytochrome *c* specific primer, complementary to codons 85-91 of the wild-type cytochrome *c* cDNA, was used to sequence the 3' end of the coding region and part of the noncoding region of the cytochrome *c* gene.



**Figure 1. Sequencing strategy of wild-type (A) and mutant (B) cytochrome *c* cDNA inserts**

The filled boxes indicate the coding regions for the apocytochrome *c* proteins, the first nucleotide of which being indicated by the number 1. The dashed lines correspond to the areas of hybridization of the synthetic oligonucleotide primers used in sequence analysis. The direction and extent of sequence determination are shown by horizontal arrows.

### 4.3 Nucleotide sequence of the wild-type cytochrome *c* cDNA

The A5 cDNA insert contains 683 bp derived from the cytochrome *c* mRNA including a poly(A) tail of 21 nucleotides. It consists of a noncoding 5' region of 105 nucleotides followed by an open-reading frame of 327 bp corresponding to a protein of 108 amino acids (Figure 2). The protein sequence deduced from the cDNA sequence is in agreement with the previously determined protein sequence for cytochrome *c* from *N. crassa* (Heller & Smith, 1966; Lederer & Simon, 1974). The 5' noncoding region contains a significantly low content of guanosine residues, only 8.5 % of total residues. The sequence GTCAA preceding the ATG initiation codon, displays similarities with many other sequences of *N. crassa* mRNAs at this position e.g. porin (Kleene *et al.*, 1987), cyclophilin (Tropschug *et al.*, 1988) the  $\beta$ -subunit of the ATP synthase complex (Rassow & Neupert, unpublished results) and the ADP/ATP carrier protein (Arends & Sebald, 1984).

Several interesting features were observed in the 3' noncoding region of the cytochrome *c* mRNA. A number of uridine-rich regions can be seen, an observation previously reported for other eucaryotic mRNAs (Boss *et al.*, 1981). The sequence AAUAAA occurring ~20 nucleotides preceding the poly(A) tail in several mRNAs, has been proposed to play an important role in polyadenylation (Proudfoot & Brownlee, 1976). In the cytochrome *c* mRNA, however, the sequence AAGAAA occurs 17 nucleotides upstream from the polyadenylation site. Thus it is assumed that this sequence represents the polyadenylation signal in *N. crassa* cytochrome *c* mRNA.

An alignment study of the *N. crassa* cytochrome *c* mRNA sequence with that of iso-1-cytochrome *c* from *Saccharomyces cerevisiae* (Boss *et al.*, 1981) reveals a high degree of homology (65 %), comparing the coding regions of the two mRNAs (results not shown). The same degree of homology was observed at the level of the amino acid sequence.



#### 4.4 Presence of an intervening sequence in the mutant cytochrome *c* mRNA

The 610 bp mutant cytochrome *c* cDNA was digested with restriction enzymes and the resulting fragments were sequenced in pUC19 (Figure 1B). Synthetic cytochrome *c* specific primers were also used to sequence the 5' and 3' regions of the insert.

The mutant sequence is identical to the wild-type sequence up to the nucleotide position 302 (not shown here). Beyond this point the mutant mRNA assumes a new sequence resulting in a total coding region of 383 nucleotides, in contrast to the 327 nucleotides of the wild-type coding region. Owing to this change in the 3' area of the coding region the mutant mRNA codes for a cytochrome *c* protein with an altered and extended carboxy terminus (19 amino acids) (Figure 3). Genomic cytochrome *c* clones from wild-type *N. crassa* were also isolated and sequenced to investigate if an intron exists at this position in the wild-type gene. A cytochrome *c* primer, hybridizing to codons 85-91 of the wild-type cDNA, was used to sequence the corresponding region of the gene. The resulting partial gene sequence is represented in an alignment study with the 3' regions of the wild-type and mutant mRNA sequence (Figure 3). Indeed, the cytochrome *c* gene contains an intron at a position corresponding to codon 101 of the wild-type cDNA sequence.

A closer look at the mutant mRNA sequence indicates that the original 3' end of the coding region of the wild-type mRNA is interrupted by an intervening sequence starting after nucleotide 302 of the wild-type sequence. This intervening sequence extends for 74 nucleotides after which the wild-type sequence is reassumed, now out of frame, at a position corresponding to the 2nd last codon of the mutant coding region. A termination codon, normally out of frame within the original wild-type coding region, is brought into frame due to the presence of this intervening sequence and serves to terminate the translation of the mutant cytochrome *c*.

The intervening sequence displays all the characteristics of an unspliced intron: the sequence GUACAU exists at the 5' insertion junction and resembles the conserved 5' intron consensus sequence GTAXGT previously reported for *Neurospora* genes (Arends & Sebald, 1984). The conserved 3' intron consensus sequence, CTPuAC...9 -

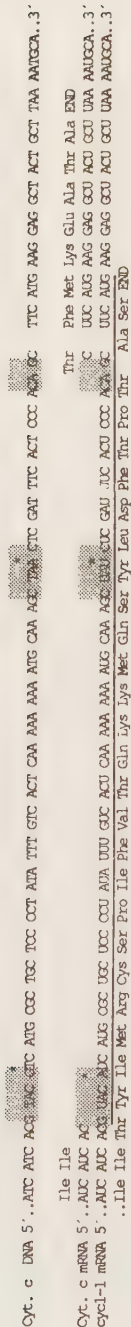


Figure 3. The mutant cytochrome *c* mRNA contains an unspliced intron.

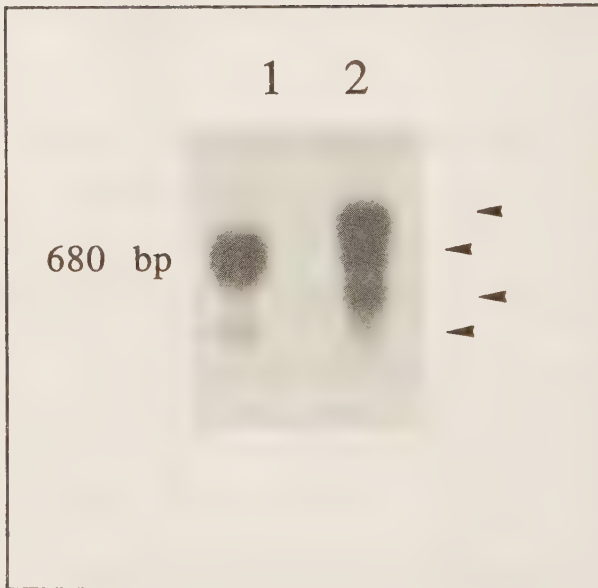
The nucleotide sequence of the 3' coding region of the wild-type cytochrome *c* gene is displayed in the upper half of the figure and is aligned with the corresponding partial nucleotide sequences of the wild-type and mutant, *cycl-1*, cytochrome *c* mRNAs, below. The amino acid sequence deduced from the wild-type mRNA is displayed above the nucleotide sequence, the first codon presented corresponds to codon 99 of the complete cytochrome *c* mRNA sequence. The nucleotide sequence and deduced amino acid sequence from the mutant mRNA is presented below that of the wild-type. The intervening sequence of the mutant mRNA is indicated by an underlined area, and asterisks indicate that two base exchanges in the intervening nucleotide sequence, which is otherwise identical to the intron sequence present in the wild-type gene. The shaded areas indicate the 5' and 3' intron consensus sequences and the branch sequence in the DNA and the mutant mRNA sequences.

15 nt . . . PyAG (Arends & Sebald, 1984) would also appear to be present since a similar sequence CUAUC is situated 14 nucleotides upstream from a CAG triplet present at the 3' insert junction. Thus the classic 5' and 3' intron consensus sequences which are present in the wild-type gene sequence are not found in the unspliced intron. In contrast, in the mutant mRNA these sequences contain two base exchanges at positions which are indicated by asterisks in Figure 3.

#### 4.5 Analysis of the wild-type and mutant *N. crassa* cytochrome *c* mRNAs

To obtain information about the cytochrome *c* transcripts, Northern blot analysis was performed with the wild-type and the *cyc1-1* poly(A)<sup>+</sup> mRNA, using the A5 cDNA insert as a labelled probe. This cytochrome *c* fragment hybridized to two mRNA species of the wild-type mRNA (Figure 4, lane 1). The larger and more abundant transcript displayed the same mobility as the full-length cDNA insert (results not shown). Southern blot analysis of both the wild-type and mutant genomic DNA has shown that *N. crassa* contains only one gene for cytochrome *c* (see below). Thus the second, smaller and less abundant cytochrome *c* mRNA must represent a second transcript of this single cytochrome *c* gene. The presence of a sequence AAUAAA in the 3' noncoding region (nucleotide position 380) could represent a second polyadenylation signal and thus result in a smaller mRNA transcript with a correspondingly shortened 3' terminus.

The nick-translated cytochrome *c*-specific probe hybridized to four mRNA species of the mutant mRNA. The two less abundant of these transcripts displayed the same mobilities as those detected in the wild-type mRNA and presumably represent normal wild-type transcripts (Figure 4, lane 2). The other two remaining transcripts are larger by the same proportion in comparison with the large and small transcripts of the wild-type mRNA, a finding which would be in good agreement with the fact that these mRNAs contain an unspliced intervening sequence. The source of the wild-type sized transcripts present in the mutant mRNA is still not clear. These transcripts may be due to the correct splicing of the mutant mRNAs with low efficiency.



**Figure 4.** Northern blot analysis of poly(A)<sup>+</sup> mRNA from wild-type and *cycl-1* *N. crassa*

Poly(A)<sup>+</sup> mRNA (10 µg) from wild-type (lane 1) and *cycl-1* (lane 2) *N. crassa* was resolved on 2.5 % agarose-formaldehyde gels, blotted onto Biotodyne filters and hybridized to a nick-translated cDNA insert, representing the full-length wild-type cytochrome *c* mRNA, see Methods.

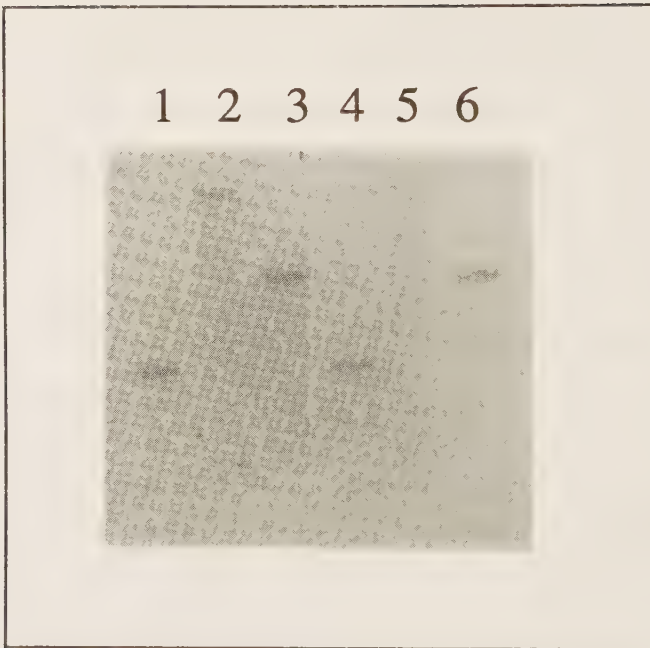
#### 4.6 Number of cytochrome *c* genes per haploid genome of *Neurospora crassa*

In order to investigate the complexity of cytochrome *c* -like sequences in *N. crassa* wild-type and *cyc1-1* mutant genomes, genomic DNA was digested with various restriction endonucleases, resolved by agarose-gel electrophoresis and analysed by Southern blotting (3.8). A full-length wild-type cytochrome *c* cDNA insert, A5, was nick translated (3.12.7) and used as a hybridization probe (Figure 5). In each lane only one hybridizing fragment was observed, indicating that only one gene for cytochrome *c* exists in both wild type and *cyc1-1* genomes.

#### 4.7 Synthesis of wild-type and mutant cytochrome *c* *in vitro*

cDNA inserts containing the complete coding regions were cloned into the *Pst*I site of the pDS5 transcription vector (Stueber *et al.*, 1984) for both the wild-type and mutant cytochrome *c*. Transcription of the cytochrome *c* cDNAs was carried out in the presence of 7mGpppA and *Escherichia coli* RNA polymerase as described (Stueber *et al.*, 1984). The resulting mRNAs were translated in rabbit reticulocyte lysates in the presence of [<sup>35</sup>S]methionine. Translation products were immunoprecipitated with cytochrome *c*-specific antiserum (Zimmermann *et al.*, 1979) and were analysed by SDS/PAGE and fluorography (Figure 6).

Transcription/translation of the wild-type cytochrome *c* cDNA yielded a product which was recognized by the cytochrome *c* antiserum (Figure 6, lane 3) and which displayed the same apparent molecular weight (12.7 kD) as apocytochrome *c* immunoprecipitated from reticulocyte lysate programmed with wild-type poly(A)<sup>+</sup> mRNA (Figure 6, lane 1). Translation of the mutant poly(A)<sup>+</sup> mRNA resulted in the synthesis of two apocytochrome *c* proteins (Figure 6, lane 2) having apparent molecular weights of 15.3 kD and 12.7 kD. The latter apocytochrome *c* displayed the same electrophoretic mobility as the wild-type apocytochrome *c*; it probably represents the translation product of the mutant mRNA correctly spliced with low efficiency.



**Figure 5.** Southern blot analysis of genomic DNA from wild-type and *cycl-1* *N. crassa*

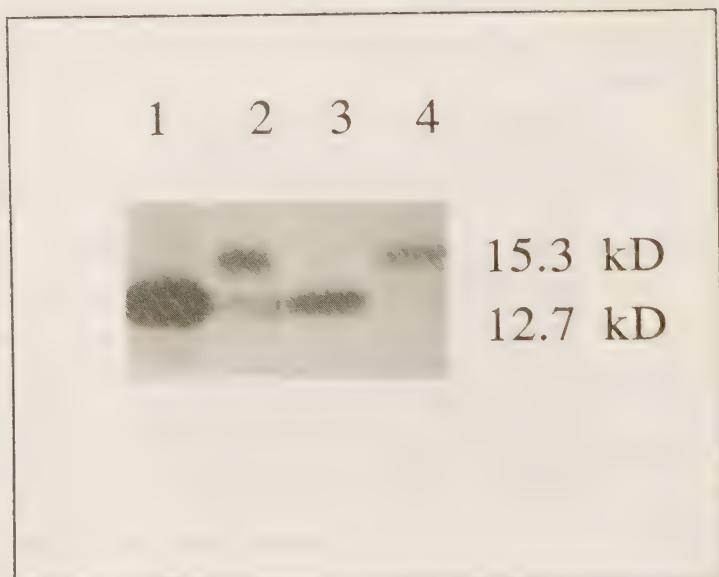
Each lane contains 10  $\mu$ g of DNA isolated from wild-type (lanes 1-3) or *cycl-1* (lanes 4-6) *N. crassa*. Samples were digested with *Hind* III (lanes 1 and 4), *Eco*RI (lanes 2 and 5) or *Pst*I (lanes 3 and 6), transferred to Biodyne filters and hybridized to the nick-translated full-length cDNA insert for the wild-type cytochrome *c*. The restriction enzymes used do not cut in the cytochrome *c* cDNA sequence.

Transcription/translation of the mutant cDNA insert resulted in the synthesis of an apocytochrome *c* with an apparent molecular weight of 15.3 kD (Figure 6, lane 4), i.e. analogous to that of the large apocytochrome *c* protein observed in the mutant poly(A)<sup>+</sup> mRNA translation. The difference in molecular weights observed between the wild-type and mutant apocytochrome *c* proteins confirmed the cDNA sequence data which indicated that the mutant mRNA coded for an apocytochrome *c* protein containing an extra 19 amino acid residues.

#### **4.8 Lack of import of *cycl-I* apocytochrome *c* into mitochondria *in vitro***

It has been demonstrated that the translocation of apocytochrome *c* across the outer mitochondrial membrane is intrinsically coupled with the covalent addition of heme; a process catalysed by the enzyme cytochrome *c* heme lyase (CCHL) (Nicholson *et al.*, 1988). Holocytochrome *c* formation is dependent on the presence of NADH and on a cytosolic factor, a flavin nucleotide FAD or FMN (Nicholson & Neupert, 1989). An assay system has been recently developed to directly measure the process of heme addition to apocytochrome *c*, thus, import of this precursor and its conversion to holocytochrome *c* can now be quantitatively demonstrated (Nicholson *et al.*, 1987a). Using this assay system, both the wild-type and mutant apocytochrome *c* species were tested for their ability to bind to wild-type mitochondria and to be converted to holocytochrome *c* upon import into mitochondria.

Transcription of both wild-type and mutant cDNA inserts in pDS5 was carried out, followed by translation of the resulting RNAs in reticulocyte lysates in the presence of [<sup>35</sup>S]cysteine. Isolated mitochondria (75 µg protein) were incubated with reticulocyte lysates either in the presence or absence of NADH, for 10 min at 25 °C. Mitochondria and a post-mitochondrial supernatant were resolved and cytochrome *c* was immunoprecipitated using an antiserum which recognized both apo- and holocytochrome *c*. Immunoprecipitates were then subjected to tryptic digestion and resulting peptide mixtures were resolved by reverse-phase HPLC and [<sup>35</sup>S]cysteine-containing peptides were collected and radioactivity quantified, as previously described (Nicholson *et al.*, 1987a).



**Figure 6.** *In vitro* synthesis of wild-type and mutant apocytochrome *c*

Poly(A)<sup>+</sup> mRNA, isolated from either the wild-type (lane 1) or *cyc1-1* mutant (lane 2) *N. crassa*, was translated in rabbit reticulocyte lysates and apocytochrome *c* was immunoprecipitated. Plasmids (pDS5) containing either wild-type (lanes 3) or mutant (lane 4) cDNA inserts for cytochrome *c* were transcribed with *E. coli* RNA polymerase in the presence of 7mGpppA. The resulting RNAs were translated in reticulocyte lysate and apocytochrome *c* was immunoprecipitated. Finally the [<sup>35</sup>S]methionine-labelled immunoprecipitates were separated by SDS/PAGE and visualized by autoradiography.

In the absence of reductants (NADH or sodium dithionite) formation of holocytochrome *c* does not occur. As a consequence, the binding of apocytochrome *c* to mitochondria could be examined independently of subsequent import steps. Binding of wild-type apocytochrome *c* to mitochondria was not prevented in the absence of NADH (Table 1). This is in agreement with previous findings of Nicholson & Neupert, 1989, who demonstrated that NADH was required for an event which is subsequent to the initial binding of apocytochrome *c* to mitochondria. In the absence of NADH only negligible wild-type holocytochrome *c* formation occurred, however, in the presence of NADH up to a 50-fold stimulation in CCHL activity was observed, in agreement with previous results (Nicholson *et al.*, 1987). The mutant apocytochrome *c* was not targeted to the mitochondria, as no significant binding of the precursor to mitochondria or conversion to holocytochrome *c* was observed, irrespective of whether NADH was present or not (Table 1).

#### 4.9 Conversion of wild-type and mutant apocytochrome *c* by solubilized CCHL

Apocytochrome *c* possesses "membrane-insertion activity" which enables it to spontaneously penetrate the outer mitochondrial membrane (Rietveld & de Kruijff, 1984; Rietveld *et al.*, 1985a, b; Dumont & Richards, 1984; Rietveld *et al.*, 1986; Berkhout *et al.*, 1987). Thus apocytochrome *c* partially inserts into the outer membrane from where it is recognized and binds with high affinity to specific binding sites in a complex which includes cytochrome *c* heme lyase (CCHL) (Nicholson *et al.*, 1988). The inability of the mutant apocytochrome *c* to bind to and consequently be imported into mitochondria could reflect either a decrease in this membrane-insertion activity and/or an inability to specifically bind to and form a stable complex with CCHL. It would appear that CCHL alone can account for both the binding of apocytochrome *c* and conversion to holocytochrome *c*. However, the possible involvement of another protein embedded in the outer membrane in binding apocytochrome *c* prior to its interaction with CCHL cannot as yet be ruled out (Nicholson *et al.*, 1988). It has been previously been

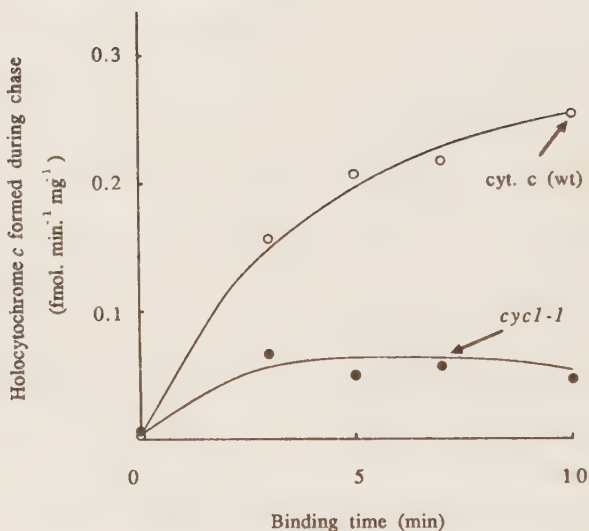
Table 1

| Binding and import of wild-type and <i>cyc1-1</i> apocytochrome <i>c</i> |                                                           |                                                 |                            |
|--------------------------------------------------------------------------|-----------------------------------------------------------|-------------------------------------------------|----------------------------|
| Sample                                                                   | Total (= 100 %) apo and holocyt. <i>c</i> in assay (pmol) | % Apocyt. <i>c</i> associated with mitochondria | % Holocyt. <i>c</i> formed |
| 1. wild-type + NADH                                                      | $9.6 \times 10^{-4}$                                      | 10                                              | 51.7                       |
| 2. wild-type - NADH                                                      | $6.0 \times 10^{-4}$                                      | 30                                              | 0.9                        |
| 3. <i>cyc1-1</i> + NADH                                                  | $4.2 \times 10^{-4}$                                      | 2.3                                             | 1.7                        |
| 4. <i>cyc1-1</i> - NADH                                                  | $2.3 \times 10^{-4}$                                      | 5.5                                             | 0.5                        |

**Table 1.** Full-length cDNA inserts for both wild-type and *cyc1-1* cytochrome *c* were transcribed and resulting transcripts were translated in reticulocyte lysates in the presence of [<sup>35</sup>S]cysteine. Isolated mitochondria (75 µg protein) were incubated with the reticulocyte lysates either in the presence or absence of NADH, for 10 min at 25 °C. Mitochondria and supernatants were separated by centrifugation and apo- and holocytochrome *c* were co-immunoprecipitated. Immunoprecipitates were eluted with urea, digested with trypsin and resulting peptide mixtures were resolved by reverse-phase HPLC. Fractions containing apo- and holopeptides were collected and radioactivity determinations were carried out by scintillation counting. For each sample, the amount of cytochrome *c* immunoprecipitated from the mitochondrial and supernatant fractions were summed together and set as 100 %. The various samples were as follows (1) wild-type apocytochrome *c* + NADH; (2) wild-type apocytochrome *c* - NADH; (3) *cyc1-1* apocytochrome *c* + NADH; (4) *cyc1-1* apocytochrome *c* - NADH.

demonstrated that association of apocytochrome *c* with CCHL can also take place after solubilizing mitochondria with octylglucoside detergent (Nargang *et al.*, 1988; Nicholson *et al.*, 1988). Using this soluble assay we could investigate independently from membrane barriers, the competence of the *cyc1-1* mutant apocytochrome *c* to serve as a substrate for the cytochrome *c* heme lyase enzyme. The binding of apocytochrome *c* to mitochondria was found to be very sensitive to the presence of salt, however, once bound to mitochondria in the absence of salt, subsequent formation of holocytochrome *c* is unaffected by the presence of upto 0.5 M salt. Identical results were obtained with the octyl glucoside solubilized CCHL enzyme (Nicholson *et al.*, 1988). That the formation of the complex of apocytochrome *c* and CCHL can be impeded by the addition of salt, enabled a kinetic analysis of the binding of both the wild-type and *cyc1-1* mutant apocytochrome *c* to the detergent solubilized CCHL to be performed. Cytochrome *c* heme lyase was solubilized from mitochondria with octyl glucoside and was incubated at 0 °C with [<sup>35</sup>S]cysteine-labelled wild-type or *cyc1-1* mutant apocytochrome *c* for the indicated time periods (Figure 7). Addition of KCl (0.3 M, end concentration) thus prevented subsequent interaction of the apocytochrome *c* with CCHL and CCHL-bound apocytochrome *c* was chased to holocytochrome by the addition of sodium dithionite. Formation of *cyc1-1* mutant holocytochrome *c* occurred at a significantly lower efficiency in comparison to wild-type holocytochrome *c* formation.

These results would suggest that the addition of extra amino acids at the carboxy terminus reduces the ability of *cyc1-1* mutant apocytochrome *c* molecule to interact specifically with cytochrome *c* heme lyase. Consequently, reduction in the levels of holocytochrome *c* present in the *cyc1-1* mitochondria was observed. The membrane-insertion activity of *cyc1-1* cytochrome *c* was also assayed (to be discussed later, 4.30), however, it was demonstrated that *cyc1-1* apocytochrome *c* still possesses the ability to spontaneously insert into the outer mitochondrial membrane.



**Figure 7 Kinetics of formation of salt-resistant complex between wild-type or *cycl-1* mutant apocytochrome *c* and cytochrome *c* heme lyase**

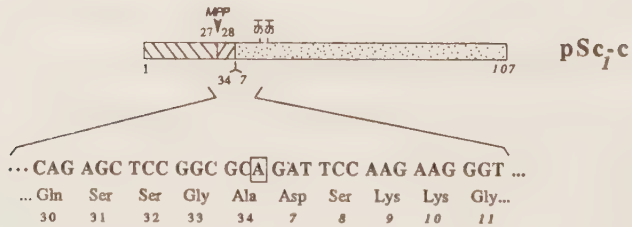
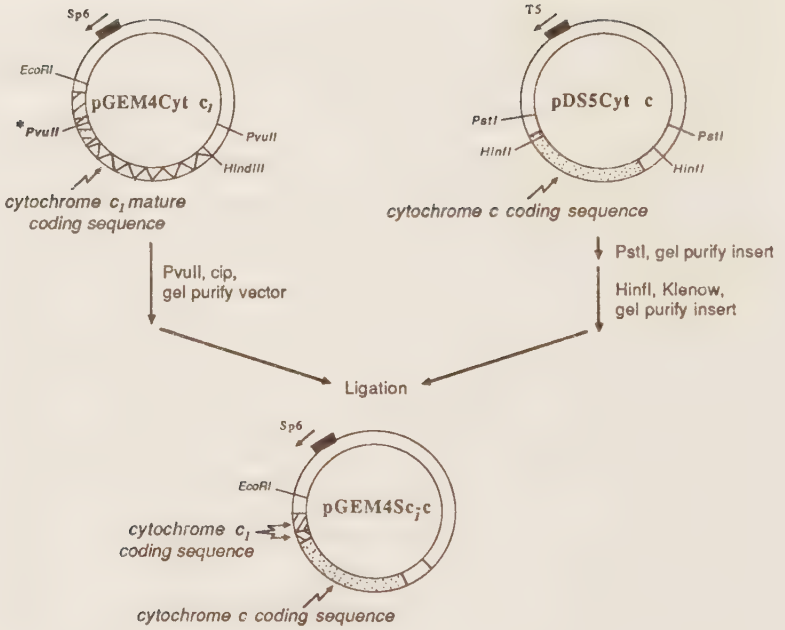
Cytochrome *c* heme lyase activity was solubilized from mitochondria at 0 °C by mixing mitochondria (at 5 mg protein/ml) with an equal volume of 2 % (w/v) *n*-octyl glucoside (Boehringer Mannheim) in SEM buffer containing 1 mM PMSF. The mixture was shaken briefly at 1 min intervals for 5 min. The preparation was centrifuged for 20 min at 27,000 × *g* (Beckman, JA20 rotor) and the upper two thirds of the resulting supernatant was removed and used immediately. Reticulocyte lysates containing [<sup>35</sup>S]cysteine-labelled wild-type or mutant apocytochrome *c* (equivalent amounts of the two precursor proteins were present) were combined with the octyl-glucoside extract of mitochondria (equivalent to 75 µg protein) in a final volume of 100 µl SEM buffer. Following incubation at 0 °C for the indicated time periods, further association of apocytochrome *c* with CCHL was blocked by the addition of 20 µl of 3 M KCl (in SEM buffer). The salt-resistant CCHL-bound apocytochrome *c* was then chased to holocytochrome *c* by the addition of hemin (3 µM), sodium dithionite (1 mg/ml) and SEM buffer to a final volume of 200 µl and incubating for a further 10 min at 25 °C. The KCl concentrations after these additions (0.3 M) was sufficient to block any further interaction between apocytochrome *c* and CCHL. Levels of holocytochrome *c* wild-type (○) or *cycl-1* mutant (●) formed during the chase period are presented.

#### 4.10 Construction of the fusion proteins pSc<sub>1</sub>-c and pLc<sub>1</sub>-c

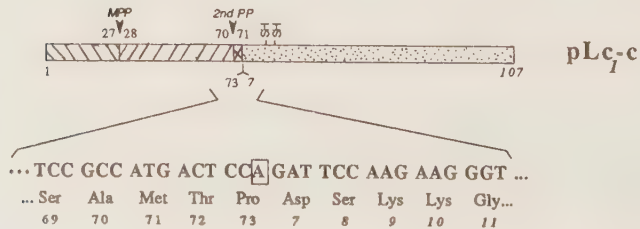
The problem as to what endogenous properties does apocytochrome *c* hold to allow it to circumvent the "conservative sorting" pathway to gain access to the mitochondrial intermembrane space by an entirely "new" pathway was then addressed. The possibility that the unique property of apocytochrome *c* to spontaneously insert into membranes confers upon the precursor protein the ability to circumvent the initial classic steps of mitochondrial import, namely, surface receptor binding and initial membrane insertion mediated by GIP, was then examined. This question was addressed by constructing two novel fusion proteins, which are composed of the matrix-targeting domain of the cytochrome *c*<sub>1</sub> presequence joined to the amino terminus of apocytochrome *c* or of the complete presequence of cytochrome *c*<sub>1</sub> joined to the apocytochrome *c*. The import pathways of these fusion proteins, which contain dual targeting information for distinct import pathways, were characterized. Furthermore, the membrane-inserting property of apocytochrome *c* was demonstrated to be able to substitute for the receptor/GIP system by directly mediating the insertion of the precursor protein into the mitochondria membrane system where the matrix-targeting domain can then respond to the membrane potential for complete translocation across the inner membrane into the matrix.

For the construction of the plasmids encoding the fusion proteins pSc<sub>1</sub>-c and pLc<sub>1</sub>-c (Figure 8), the *EcoRI/HindIII* fragment of *N. crassa* cytochrome *c*<sub>1</sub> cDNA, which encodes the 5' noncoding region of the amino-terminal half of the cytochrome *c*<sub>1</sub> protein (Römisch *et al.*, 1987), was cloned into the plasmid pMa5-8 (Fritz *et al.*, 1986). Oligonucleotide site-directed mutagenesis (3.14) was performed to introduce a unique *PvuII* site in the cytochrome *c*<sub>1</sub> cDNA sequence by changing a C residue for an A residue either at a position corresponding to codon 34 (for the pSc<sub>1</sub>-c fusion protein) or at a position corresponding to codon 73 (for the pLc<sub>1</sub>-c fusion protein). DNA restriction analysis and sequencing was performed to verify correct mutagenesis. The mutagenized *EcoRI/HindIII* fragments were cloned into the polylinker of the plasmid pGEM4. The recombinant plasmids were then subsequently cleaved with *PvuII* and treated with calf intestinal phosphatase (3.12.4) and purified by electroelution (3.10.1).

A



B



**Figure 8. Construction of pSc<sub>I</sub>-c and pLc<sub>I</sub>-c, fusion proteins between cytochrome c<sub>1</sub> and cytochrome c**

**A:** Details of the construction of the pSc<sub>I</sub>-c fusion protein are described in Methods. The unique *Pvu*II site introduced into the cytochrome c<sub>1</sub> cDNA sequence by site-directed mutagenesis is indicated by an asterisk. pSc<sub>I</sub>-c is a fusion protein between the first 34 amino acid residues of the cytochrome c<sub>1</sub> presequence and the complete sequence of cytochrome c, with the exception of the initial six amino acids of the amino terminus. This polypeptide is represented by a bar in the lower part of the panel A. Hatched areas indicate sequences of cytochrome c<sub>1</sub> origin while dotted areas are of cytochrome c origin. Amino acids 1 through 27 represent the first portion of the cytochrome c<sub>1</sub> presequence that is removed by the matrix processing peptidase (MPP) which cleaves between amino acids 27 and 28. Amino acids 28 through to 34 represent a small portion of the second part of the cytochrome c<sub>1</sub> presequence (7 amino acids). Amino acid residues 7 through 107 represent the cytochrome c part of the fusion polypeptide. Cysteine sulphydryl residues to which the heme becomes attached are also indicated (-SH). The region around the fusion join is enlarged and details of the coding sequence, amino acid sequence and amino acid numbering are indicated. The nucleotide introduced by the mutagenesis is indicated by a box.

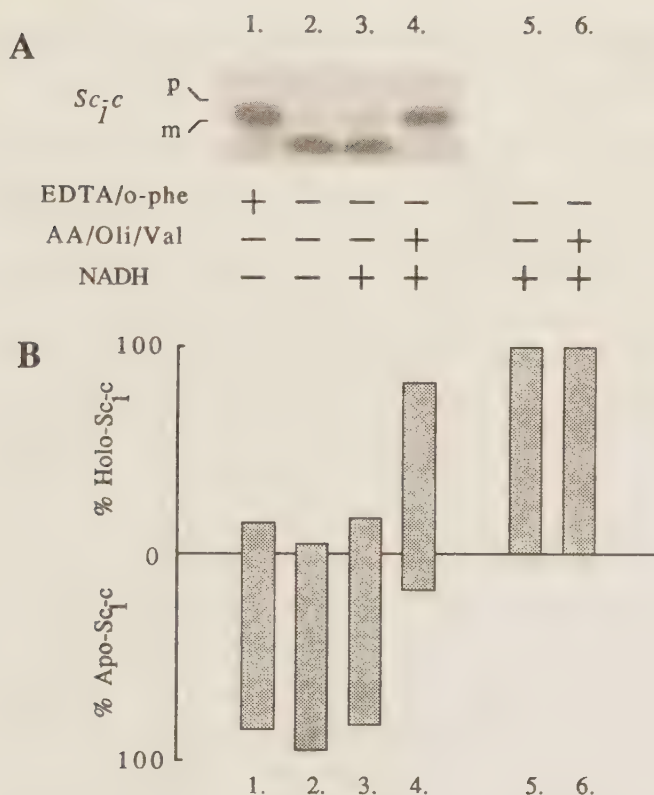
**B:** Details of the construction of pLc<sub>I</sub>-c are outlined in Methods and pLc<sub>I</sub>-c is represented in panel B also by a bar which is drawn in the same manner as pSc<sub>I</sub>-c in panel A. pLc<sub>I</sub>-c is a fusion protein consisting of the entire presequence of cytochrome c<sub>1</sub> plus three amino acids of the mature sequence, fused to a complete sequence of cytochrome c, with the exception of the initial six amino acids of the amino terminus. The sites of processing by MPP and a second processing peptidase (2nd PP) are indicated.

A *Pst* I cDNA insert, containing the complete coding region of cytochrome *c* was isolated following cleavage of the corresponding pDS5 plasmid (4.1). The purified insert was then further cleaved with *Hinf* I (which cuts the insert at a position corresponding to codon 7 and again in the 3' noncoding region) and was treated with the Klenow fragment of the *E. coli* DNA polymerase I. The resulting insert was purified and ligated into the pGEM4 vectors described above to produce in-frame fusions between the cytochrome *c*<sub>1</sub> and cytochrome *c* fragments. DNA sequencing analysis was carried out in order to verify the postulated nucleotide sequences at the join regions. The corresponding amino acid sequences, which contain no introduced residues, for both the pSc<sub>1</sub>-*c* and pLc<sub>1</sub>-*c* fusion proteins are depicted in Figure 8 A and B, respectively. Both the pSc<sub>1</sub>-*c* and pLc<sub>1</sub>-*c* fusion proteins were synthesized in reticulocyte lysate following transcription of the plasmids with SP6 RNA polymerase, as described previously (3.15.2). Both of the fusion proteins synthesized in the reticulocyte lysate could be quantitatively immunoprecipitated with an antiserum directed against both apo- and holocytochrome *c*.

#### 4.11 Selective import of the pSc<sub>1</sub>-*c* hybrid protein into mitochondria along two distinct routes

Unlike cytochrome *c* import, import of cytochrome *c*<sub>1</sub> into mitochondria is dependent on the presence of a membrane potential ( $\Delta\Psi$ ) across the inner membrane. Formation of holocytochrome *c* from apocytochrome *c* on the other hand, is dependent on NADH for the reduction of heme. We could, therefore, selectively activate either the cytochrome *c*<sub>1</sub> import pathway by the presence of  $\Delta\Psi$ , or the pathway for cytochrome *c* import by the presence of NADH or sodium dithionite.

The precursor of the Sc<sub>1</sub>-*c* fusion protein (pSc<sub>1</sub>-*c*) was synthesized in the presence of [<sup>35</sup>S]cysteine in a reticulocyte lysate that was programmed with pSc<sub>1</sub>-*c* specific transcripts obtained from the corresponding pGEM4 clone. For import of the precursor, reticulocyte lysate and isolated mitochondria were combined and incubated under different conditions at 25 °C. Import into the interior of the mitochondria was demonstrated by the resistance of the Sc<sub>1</sub>-*c* species in the mitochondria to externally added proteinase K. Following protease treatment, samples were divided and analysed



**Figure 9. Import of p*Sc<sub>I</sub>-c* into mitochondria**

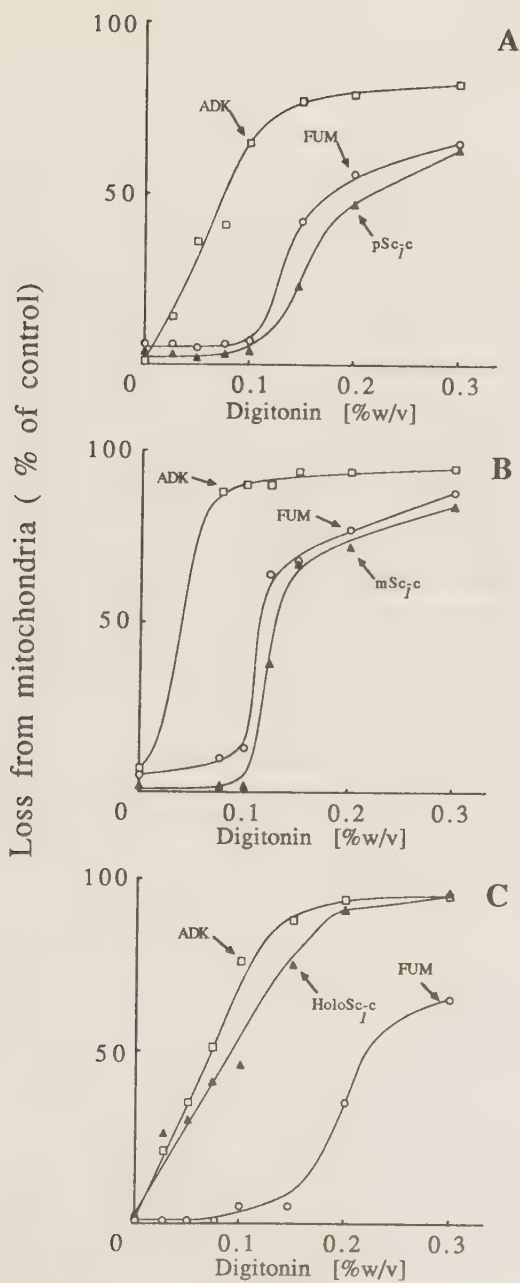
The precursor of *Sc<sub>I</sub>-c* was synthesized *in vitro* in the presence of [<sup>35</sup>S]cysteine and imported into mitochondria (100 µg protein) at 25 °C in the presence of 5 mM EDTA plus 0.2 mM *o*-phenanthroline (track 1), 2.5 mM MgCl<sub>2</sub> (tracks 2, 3 and 4), 5 mM NADH (tracks 3 and 4) or antimycin A (AA), oligomycin (Oli) and valinomycin (Val) (track 4) as described in Methods. Following proteinase K treatment, samples were lysed in 1 ml of 1 % (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris/HCl (pH 7.2) containing 2 mM PMSF. Samples were divided in half and processed as follows: *Sc<sub>I</sub>-c* was immunoprecipitated from both sets of samples, however, one set was prepared for SDS/PAGE and the resulting fluorographs are depicted in A. The remaining set of immunoprecipitates were dissociated with urea, digested with trypsin and subjected to reverse-phase HPLC. The amounts of apo- and holo-*Sc<sub>I</sub>-c* were quantified by collecting the corresponding peptides and determining their radioactivity (B). [<sup>35</sup>S]Cysteine-labelled apocytochrome *c* was incubated with mitochondria (50 µg protein) at 25 °C in the presence of 5 mM NADH (tracks 5 and 6) or antimycin A, oligomycin, and valinomycin (track 6). Following proteinase K treatment, levels of holocytochrome *c* formed were assayed (as described above for *Sc<sub>I</sub>-c*) by immunoprecipitation and subsequent HPLC analysis.

either by HPLC analysis, to allow quantitation of apo- and holocytochrome *c* levels or by SDS/PAGE in order to determine the levels of pSc<sub>J</sub>-*c* and that processed to mSc<sub>J</sub>-*c*.

In the presence of  $\Delta\Psi$ , the pSc<sub>J</sub>-*c* precursor was imported into mitochondria and was processed to its mature-size form, mSc<sub>J</sub>-*c* (Figure 9A, lane 2). When the chelators EDTA and *o*-phenanthroline were added to the import reaction, in order to inhibit the matrix-located processing peptidase, Sc<sub>J</sub>-*c* could be accumulated in mitochondria as its precursor form pSc<sub>J</sub>-*c* (Figure 9A, lane 1). Both of these species, namely, pSc<sub>J</sub>-*c* and mSc<sub>J</sub>-*c*, which accumulated in the mitochondria in a  $\Delta\Psi$ -dependent fashion, were of the heme-free apocytochrome *c* form (Figure 9B, tracks 1 and 2). In the absence of  $\Delta\Psi$ , import of pSc<sub>J</sub>-*c* did not occur unless NADH was present. Under these conditions (i.e.  $-\Delta\Psi$ , +NADH) the cytochrome *c* pathway alone, was active and a pSc<sub>J</sub>-*c* species (Figure 9A, lane 4) to which heme was attached (Figure 9B, track 4) accumulated in the mitochondria. When both the cytochrome *c*<sub>1</sub> (matrix-import pathway) and the cytochrome *c* pathway were active (i.e. +  $\Delta\Psi$ , +NADH), the matrix route was dominant; larger amounts of protease-resistant mSc<sub>J</sub>-*c* were accumulated than pSc<sub>J</sub>-*c* (Figure 9A, lane 3). Reduction of import along the cytochrome *c* route under these conditions was also reflected by the low levels of holo-pSc<sub>J</sub>-*c* that were synthesized (Figure 9B, track 3). Thus formation of holo-pSc<sub>J</sub>-*c* was favoured under conditions where  $\Delta\Psi$  was absent (i.e. when the matrix route was inactive) in contrast to holocytochrome *c* formation which was entirely independent of  $\Delta\Psi$  (Figure 9B, tracks 5 and 6).

#### **4.12 Submitochondrial localization of imported pSc<sub>J</sub>-*c* species by digitonin treatment of mitochondria**

It would appear from these results that the pSc<sub>J</sub>-*c* fusion protein could be selectively imported either along the matrix- or the cytochrome *c*-import pathways. If indeed this was the case, we would expect pSc<sub>J</sub>-*c* and mSc<sub>J</sub>-*c* imported in a  $\Delta\Psi$ -dependent manner to be located in the mitochondrial matrix. Holo-pSc<sub>J</sub>-*c* should, on the other hand, be located in the intermembrane space. To determine the submitochondrial localization of these various Sc<sub>J</sub>-*c* species, import of [<sup>35</sup>S]cysteine-labelled pSc<sub>J</sub>-*c* into mitochondria was performed either in the presence of  $\Delta\Psi$  and EDTA/*o*-phenanthroline (Figure 10A) or



**Figure 10. Submitochondrial localization of imported *Sc<sub>J</sub>-c* species by digitonin treatment of mitochondria**

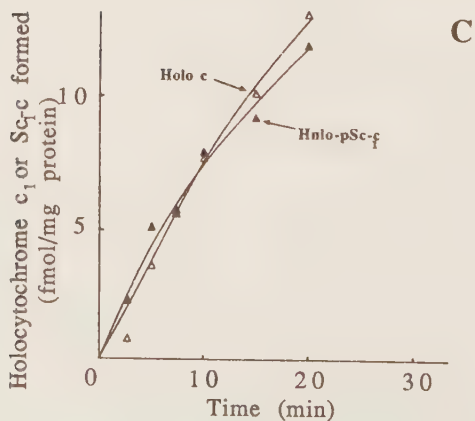
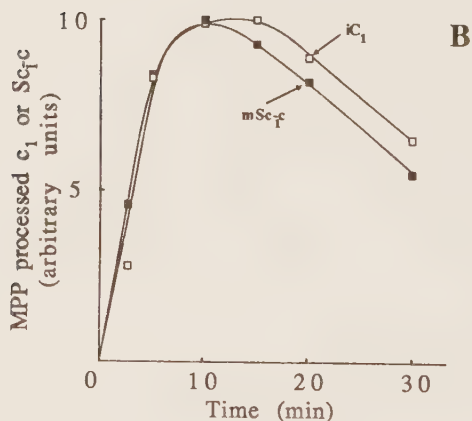
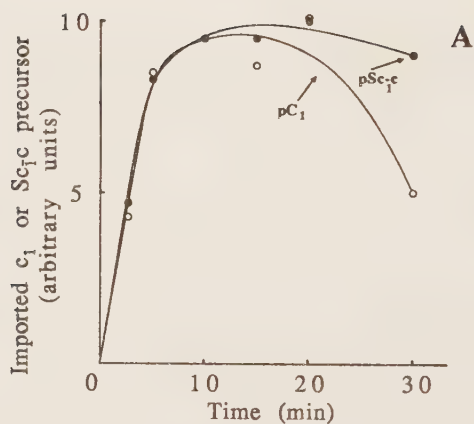
The [<sup>35</sup>S]cysteine-labelled p*Sc<sub>J</sub>-c* was imported into mitochondria (675 µg protein, 1.35 ml final volume) at 25 °C in the presence of 5 mM EDTA plus 0.2 mM *o*-phenanthroline (panel A), 2.5 mM MgCl<sub>2</sub> (panel B), or 5 mM NADH plus antimycin A, oligomycin and valinomycin (panel C). Following proteinase K treatment, mitochondria were reisolated and resuspended in SEM-buffer containing 100 mM KCl (SEMK) and divided into aliquots. Digitonin was added to concentrations of 0 to 0.3 % (w/v) and incubated for 2 min at 0 °C in a final volume of 20 µl and at a protein concentration of 5 mg/ml. Afterwards, reactions were diluted 20-fold with SEMK and were separated into pellet and supernatant fractions by centrifugation for 10 min at 27,000 × *g* (Beckman, JA-20 rotor). In the case of holo-p*Sc<sub>J</sub>-c* (Panel C), prior to centrifugation, samples were treated with proteinase K, (60 µg/ml, for 30 min at 0 °C). PMSF was then added to inhibit further proteolytic activity. These samples were then treated in an identical manner as those in Panels A and B, which was as follows. Supernatants were adjusted to 1 % (w/v) Genapol and the pellets were resuspended in SEMK plus 1 % (w/v) Genapol, to the same volume as the supernatants. Both supernatant and pellet fractions were divided in half. *Sc<sub>J</sub>-c* was immunoprecipitated (see Methods) from one set of samples and analysed either by SDS/PAGE, where the resulting fluorographs were quantified by laser densitometry (panel A and B, ▲) or the immunoprecipitates were digested with trypsin and subjected to reverse-phase HPLC. The holo-p*Sc<sub>J</sub>-c* peptides were collected and quantified by determining their radioactivity (panel C). The remaining parts of the samples were used for determination of marker enzyme activities; adenylate kinase (□) and fumarase (○). For each digitonin concentration the activities recovered from the pellet and supernatant fractions were set to 100 %. The loss from the mitochondria is plotted. ADK, adenylate kinase; FUM, fumarase.

$\Delta\Psi$  alone (Figure 10B) or in the presence of NADH but absence of  $\Delta\Psi$  (Figure 10C). Mitochondria were reisolated and treated with increasing concentrations of digitonin to sequentially open the intermembrane space and matrix compartments, as judged by the release of the respective marker enzymes, adenylate kinase and fumarase.

When the matrix-import pathway alone was active (i.e.  $+\Delta\Psi$ ), import pSc<sub>I</sub>-c (Figure 10A) and mSc<sub>I</sub>-c (Figure 10B) were released by subsequent digitonin treatment in a manner very similar to fumarase. This indicated that the imported pSc<sub>I</sub>-c and mSc<sub>I</sub>-c were soluble species and that they were located in the mitochondrial matrix. When the import of pSc<sub>I</sub>-c was directed along the cytochrome *c* import route (i.e.  $-\Delta\Psi$ , + NADH) the holo-pSc<sub>I</sub>-c formed was resistant to added protease which showed that it was translocated into the mitochondria, however, it was found to be only partially soluble after digitonin treatment of the mitochondria (results not shown). Apparently the presequence leads to an interaction of the uncleaved holo-form of the precursor with the mitochondrial membrane. Therefore, fractionation of the mitochondria with digitonin was combined with protease treatment. Holo-pSc<sub>I</sub>-c was digested as soon as the intermembrane space was opened (Figure 10C). This demonstrated that the holo-pSc<sub>I</sub>-c was localized in the intermembrane space.

#### 4.13 Kinetics of pSc<sub>I</sub>-c import into the matrix and of holo-pSc<sub>I</sub>-c formation

It has recently been described that non-specific (i.e. non-mitochondrial) targeting signals appear to use a bypass pathway for import into mitochondria (Pfaller *et al.*, 1989; Pfanner *et al.*, 1988a). The kinetics and efficiencies of this bypass import are significantly reduced in comparison to that of legitimate mitochondrial precursor proteins. It was essential, therefore, to exclude the possibility that the import of pSc<sub>I</sub>-c described above was a result of a low-efficiency non-specific pathway(s). The kinetics of accumulation of the precursor form and the intermediate-size form of cytochrome *c*<sub>1</sub> (pC<sub>1</sub> and iC<sub>1</sub>, respectively) for direct comparison. Likewise, the kinetics of holo-pSc<sub>I</sub>-c formation was compared to that of holocytochrome *c* formation from apocytochrome *c* (Figure 11 C). Accumulation of matrix-localized pSc<sub>I</sub>-c occurred with the same kinetics



### Figure 11 Kinetics of pSc<sub>I</sub>-c import into the matrix and of holo-pSc<sub>I</sub>-c formation

The cytochrome *c*, cytochrome *c*<sub>1</sub> and Sc<sub>I</sub>-*c* precursors were synthesized *in vitro* in reticulocyte lysates and were imported into mitochondria under the conditions described for Figure 9 except that the incubation periods were varied.

A. Accumulation of protease-resistant precursor-size cytochrome *c*<sub>1</sub> and Sc<sub>I</sub>-*c* (pC<sub>1</sub> and pSc<sub>I</sub>-*c*): Mitochondria (50 µg protein) were incubated with [<sup>35</sup>S]methionine-labelled precytochrome *c*<sub>1</sub> (○) or pSc<sub>I</sub>-*c* (●) at 25 °C for the indicated time periods in the presence of EDTA plus *o*-phenanthroline. Protease-resistant pC<sub>1</sub> and pSc<sub>I</sub>-*c* was then determined (see Methods) following resolution by SDS/PAGE. Cytochrome *c*<sub>1</sub> and Sc<sub>I</sub>-*c* bands from the fluorographs of resulting gels were quantified by laser densitometry.

B. Accumulation of protease-resistant processed cytochrome *c*<sub>1</sub> and Sc<sub>I</sub>-*c* (iC<sub>1</sub> and mSc<sub>I</sub>-*c*): Import of precytochrome *c*<sub>1</sub> (□) and pSc<sub>I</sub>-*c* (■) into mitochondria was carried out essentially as described above (A) except in the presence of 2.5 mM MgCl<sub>2</sub>, instead of chelators. After incubation for the indicated time periods at 25 °C, samples were processed exactly as above (A). MPP, matrix processing peptidase.

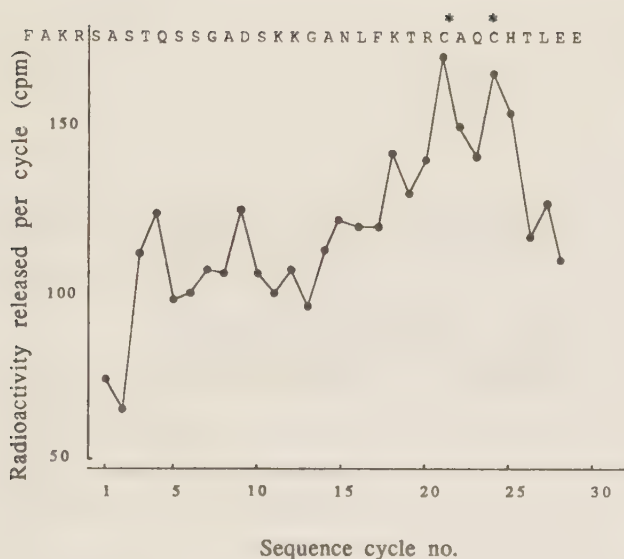
C. Formation of holocytochrome *c* and holo-pSc<sub>I</sub>-*c* in the presence of NADH: [<sup>35</sup>S]cysteine-labelled apocytochrome *c* and apo-pSc<sub>I</sub>-*c* were incubated (separately) with mitochondria (50 µg protein), in the presence of antimycin A, oligomycin and valinomycin and 5 mM NADH at 25 °C for the indicated time periods. Samples were cooled to 0 °C and were lysed with 1 ml of 1 % (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris-HCl, pH 7.2 and cytochrome *c* and Sc<sub>I</sub>-*c* were immunoprecipitated (see Methods). Following dissociation of the immunocomplex with urea, peptides were generated by digestion with trypsin and resolved by reverse-phase HPLC. The amounts of holocytochrome *c* (Δ) and holo-pSc<sub>I</sub>-*c* (▲) were quantified by collecting the corresponding peptides and determining their radioactivity.

as for pC<sub>1</sub> accumulation (Figure 11A). Similarly, import and processing to mSc<sub>1</sub>-c by the matrix-located processing peptidase also displayed kinetics similar to iC<sub>1</sub> accumulation (Figure 11B). Formation of holo-pSc<sub>1</sub>-c (i.e. along the cytochrome *c* import route) occurred with identical kinetics as the conversion of apocytochrome *c* to holocytochrome *c* (Figure 11C).

We therefore conclude that the pSc<sub>1</sub>-c hybrid protein, which contains targeting information for two diversely different pathways, can be selectively imported along either route. Furthermore, import of pSc<sub>1</sub>-c along either the matrix- or cytochrome *c*-import pathways occurs with comparable efficiency and kinetics as those of the corresponding legitimate parent precursors.

#### 4.14 Determination of the matrix processing peptidase cleavage site of the cytochrome c<sub>1</sub> presequence

The pSc<sub>1</sub>-c fusion protein was synthesized in reticulocyte lysate in the presence of [<sup>35</sup>S]cysteine in order to radiolabel the cysteine amino acid residues in positions 47 and 50. The [<sup>35</sup>S]-labelled Sc<sub>1</sub>-c precursor was imported into isolated mitochondria as described in Figure 8 in the presence of MgCl<sub>2</sub>. Protease resistant mature-size (mSc<sub>1</sub>-c) was purified by immunoprecipitation. Radiosequence analysis by automated solid-phase Edman degradation was carried out (Wachter *et al.*, 1973). Radioactivity was released from the solid-phase support following cycles 21 and 23 of Edman degradation. The amino acid sequence of the cytochrome c<sub>1</sub> presequence is aligned over the corresponding sequencer cycles in Figure 12. The proposed cleavage site determined by this method occurs between amino acids 27 and 28 of the cytochrome c<sub>1</sub> presequence and is also indicated in Figure 12 by an arrowhead. The actual site of cleavage "Arg Ser ^ Ala Ser" by the matrix processing peptidase (MPP) bears a significant degree of similarity with that of other MPP cleavage sites previously determined. The MPP cleavage site of the cytochrome c<sub>1</sub> presequence, as with many other MPP cleavage sites, supports the "minus two rule", namely, that a hydrophobic amino acid occurs at the -1 position and a positive amino acid occurs at position -2, relative to the cleavage site (Nicholson and Neupert, 1987).



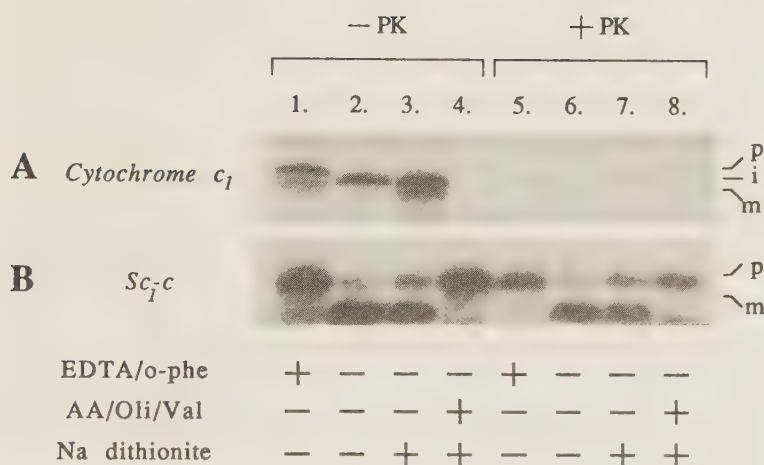
**Figure 12. Determination of the MPP cleavage site of the cytochrome  $c_1$  presequence .**

The precursor of the  $Sc_1$ - $c$  fusion protein was synthesized *in vitro* in the presence of [ $^{35}$ S]cysteine and imported into mitochondria (100  $\mu$ g protein) at 25 °C in the presence of 2.5 mM  $MgCl_2$ . Following proteinase K treatment,  $Sc_1$ - $c$  was immunoprecipitated. The mature-size  $Sc_1$ - $c$  was the only species to accumulate under these import conditions (results not shown). The  $mSc_1$ - $c$  was eluted from the protein A-Sepharose and radiosequencing analysis by automated solid-phase Edman degradation was performed (Wachter et al., 1973). The area of the cytochrome  $c_1$  presequence containing the proposed MPP cleavage site and its join with the cytochrome  $c$  sequence is depicted using the single letter amino acid code. The radio-labelled cysteine residues at positions 47 and 50 of the  $pSc_1$ - $c$  protein, are indicated by asterisks and the proposed cleavage site (between amino acids 27 and 28) is indicated by an arrowhead.

#### 4.15 Analysis of the cytochrome $c_1$ receptor requirement for the import of pSc<sub>I</sub>-c into the mitochondria

Mitochondria that are pretreated with low levels of proteases exhibit nearly normal levels of cytochrome  $c$  import (Nicholson *et al.*, 1988). This observation strongly suggests that, unlike all other mitochondrial precursor proteins studied to date (Zwizinski *et al.*, 1983; Pfaller & Neupert, 1987; Pfaller *et al.*, 1988), apocytochrome  $c$  binding and import is not mediated by protease-sensitive components on the surface of mitochondria (Nicholson *et al.*, 1988). This removal of the cytochrome  $c_1$  receptor by mild proteinase K pretreatment of mitochondria enabled us to selectively inactivate the initial recognition step of the cytochrome  $c_1$  import pathway, while at the same time leaving the entire cytochrome  $c$  pathway intact.

Mitochondria pretreated with proteinase K (50  $\mu\text{g/ml}$ ) were tested under various conditions for their ability, in comparison to untreated mitochondria, to import cytochrome  $c_1$  and pSc<sub>I</sub>-c (Figure 13, lanes 5-8 vs 1-4, respectively). In the control mitochondria the cytochrome  $c_1$  precursor (pC<sub>1</sub>) was imported into the mitochondrial matrix (Figure 13A, lane 1) where it underwent processing to intermediate-size cytochrome  $c_1$  (iC<sub>1</sub>) (Figure 13A, lane 2) and was retranslocated back across the inner membrane (Hartl *et al.*, 1987). In the presence of sodium dithionite, conversion to holocytochrome  $c_1$  took place followed by a second event (Nicholson *et al.*, 1989) which resulted in the formation of mature-size (holo) cytochrome  $c_1$  (mC<sub>1</sub>) (Figure 13A, lane 4). Removal of the cytochrome  $c_1$  receptor by treatment of the mitochondria with proteinase K abolished cytochrome  $c_1$  import (Figure 13A, lane 5-8). In the absence of the cytochrome  $c_1$  receptor import of pSc<sub>I</sub>-c along the cytochrome  $c$  pathway occurred (Figure 13B, lanes 8 vs 4) as expected, but more interestingly, efficient import of pSc<sub>I</sub>-c into the matrix, where it was processed to mSc<sub>I</sub>-c, also occurred (Figure 13B, lanes 5 vs 1 and lanes 6 vs 2).



**Figure 13. Removal of the cytochrome  $c_1$  receptor by protease treatment of mitochondria does not affect the import of  $pScI\text{-}c$  into the matrix**

Freshly isolated mitochondria were treated with proteinase K at a concentration of 50  $\mu\text{g/ml}$  as described in Methods. Control mitochondria were processed in an identical manner except that no protease was present. The resulting mitochondrial preparations were tested for their ability to import [ $^{35}\text{S}$ ]methionine-labelled precytochrome  $c_1$  (A) or  $pScI\text{-}c$  (B), essentially as described for Figure 2. Import into control mitochondria (lanes 1-4) and protease-treated mitochondria (lanes 5-8) was performed in the presence of 5 mM EDTA plus 0.2 mM *o*-phenanthroline (lanes 1 and 5), 2.5 mM  $\text{MgCl}_2$  (lanes 2-4 and 6-8), sodium dithionite (1 mg/ml) (lanes 3, 4, 7, and 8) or a mix of antimycin A (AA), oligomycin (Oli) and valinomycin (Val) (lanes 4 and 8). Protease-resistant species were analysed by SDS/PAGE and fluorography. The bands corresponding to cytochrome  $c_1$  and  $ScI\text{-}c$  in the resulting film are shown.

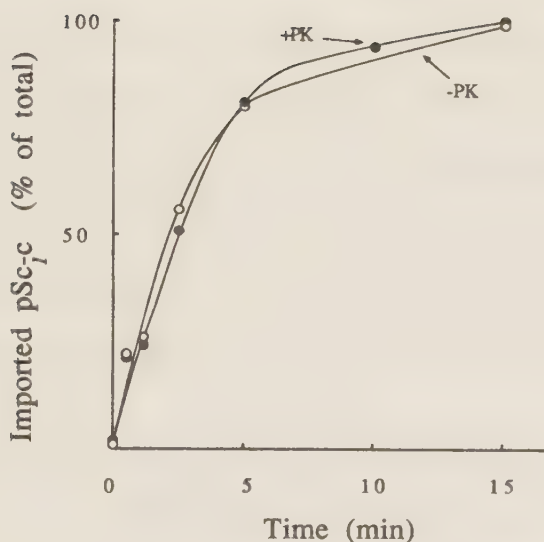
#### 4.16 Kinetics of pSc<sub>I</sub>-c import into the matrix of protease pretreated mitochondria

Binding and import of most mitochondrial precursor proteins is mediated by proteinaceous receptor sites on the mitochondrial surface (Zwizinski *et al.*, 1983, 1984; Pfaller *et al.*, 1988). Removal of these receptors by mild proteolytic treatment, severely reduces the efficiency of mitochondrial protein import. The residual import (upto 5%), however, still retains specific characteristics of normal protein import, namely, initial insertion into the outer membrane, dependence on GIP,  $\Delta\Psi$  and ATP (Pfaller *et al.*, 1988). Apart from the reduction in efficiency, the kinetics of this "by-pass" import are also significantly reduced. Import of pSc<sub>I</sub>-c into protease-treated mitochondria was shown to occur with a similar efficiency as import into untreated mitochondria (c.f. Figure 13B). The kinetics of accumulation of mSc<sub>I</sub>-c in the mitochondrial matrix of protease-treated mitochondria was then determined in parallel to those in untreated mitochondria (Figure 14). Import of pSc<sub>I</sub>-c and processing to mSc<sub>I</sub>-c by the matrix-located processing peptidase in both protease-treated and untreated mitochondria occurred with very similar kinetics.

Taken together, these results would indicate that the presence of the cytochrome *c*<sub>1</sub> receptor does not enhance either the efficiency or the kinetics of pSc<sub>I</sub>-c import into the mitochondrial matrix. It can also be concluded that properties of pSc<sub>I</sub>-c hybrid protein (specifically the cytochrome *c* domain or components of the cytochrome *c* import pathway facilitates the cytochrome *c*<sub>1</sub> receptor-independent import of pSc<sub>I</sub>-c into the mitochondrial matrix.

#### 4.17 Analysis of the requirement of GIP for pSc<sub>I</sub>-c import into the mitochondrial matrix

It has recently been reported that precursor proteins that are bound specifically to receptors on the mitochondrial surface are subsequently transferred to a component in the outer membrane called GIP, "general insertion protein", (Pfaller *et al.*, 1988). Transfer of precursors to this common insertion site can be competed for by water-soluble porin



**Figure 14. Kinetics of pScI-c import into the matrix of protease-treated mitochondria**

The ScI-c precursor protein was synthesized *in vitro* in the presence of [<sup>35</sup>S]methionine. The resulting lysate (10  $\mu$ l) was mixed with isolated mitochondria (10  $\mu$ g protein) (which had been either pretreated (●, + PK) or not (○, - PK) with proteinase K) in the presence of 2.5 mM MgCl<sub>2</sub> in a final volume of 100  $\mu$ l, and was incubated for the times indicated at 25 °C. The incubation was stopped by the addition of SME containing 0.3 M KCl, and subsequent centrifugation at 17,400  $\times$  g for 12 min (Beckman, JA-20 rotor). Import into the matrix was monitored by mScI-c formation. The mitochondrial pellets were lysed in sample buffer and resolved by SDS/PAGE. The bands corresponding to mScI-c from the fluorograph of the resulting gel were quantified by laser densitometry.

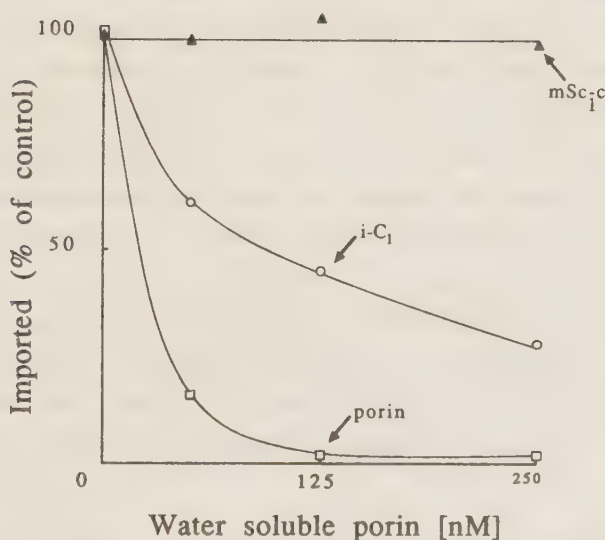
(ws porin). Competition studies with ws porin can therefore be used for determining whether GIP is involved in the import of other precursor proteins or not. The question of whether the receptor-independent import of pSc<sub>I</sub>-c into the matrix was in fact also GIP-independent was thus addressed.

Water-soluble porin was prepared (Pfaller & Neupert, 1987) and tested for its ability to compete for the integration of [<sup>35</sup>S]porin into the outer membrane, as a control and for the import of precytochrome *c*<sub>1</sub> and pSc<sub>I</sub>-c into the mitochondrial matrix (Figure 15). Water-soluble porin not only competed for the import of porin into the mitochondria but also for the import of cytochrome *c*<sub>1</sub> (as judged by iC<sub>1</sub> accumulation). Half-maximal inhibition of cytochrome *c*<sub>1</sub> import occurred at a concentration of ws porin of 90 nM, thus indicating that cytochrome *c*<sub>1</sub> import into the matrix was dependent on GIP. Import into the matrix (as measured by mSc<sub>I</sub>-c accumulation), however, was clearly not competed by ws porin.

Taken together, these data indicate that pSc<sub>I</sub>-c can be imported into the mitochondrial matrix independent of both the cytochrome *c*<sub>1</sub> receptor and the common insertion site (GIP), both of which are essential for cytochrome *c*<sub>1</sub> import.

#### **4.18 Requirement of a functional cytochrome *c* import machinery for the receptor/GIP-independent import of pSc<sub>I</sub>-c into the mitochondrial matrix**

Is the observed receptor/GIP-independent import of pSc<sub>I</sub>-c into the matrix somehow assisted by components of the cytochrome *c* import pathway, for example cytochrome *c* heme lyase (CCHL)? It has previously been proposed that CCHL alone may account for both major stages of cytochrome *c* import, namely, specific binding of apocytochrome *c* and its conversion to holocytochrome *c* (Nicholson *et al.*, 1988). Therefore in order to examine the possible role of CCHL in the import of pSc<sub>I</sub>-c into the matrix, two approaches were taken. First, competition for cytochrome *c* import components was tested using unlabelled apocytochrome *c* to see if the receptor-independent import of pSc<sub>I</sub>-c into the matrix was impeded. Second, mitochondria isolated from a CCHL-



**Figure 15 Import of pSc<sub>I</sub>-c into mitochondrial matrix is not competed for by water-soluble porin**

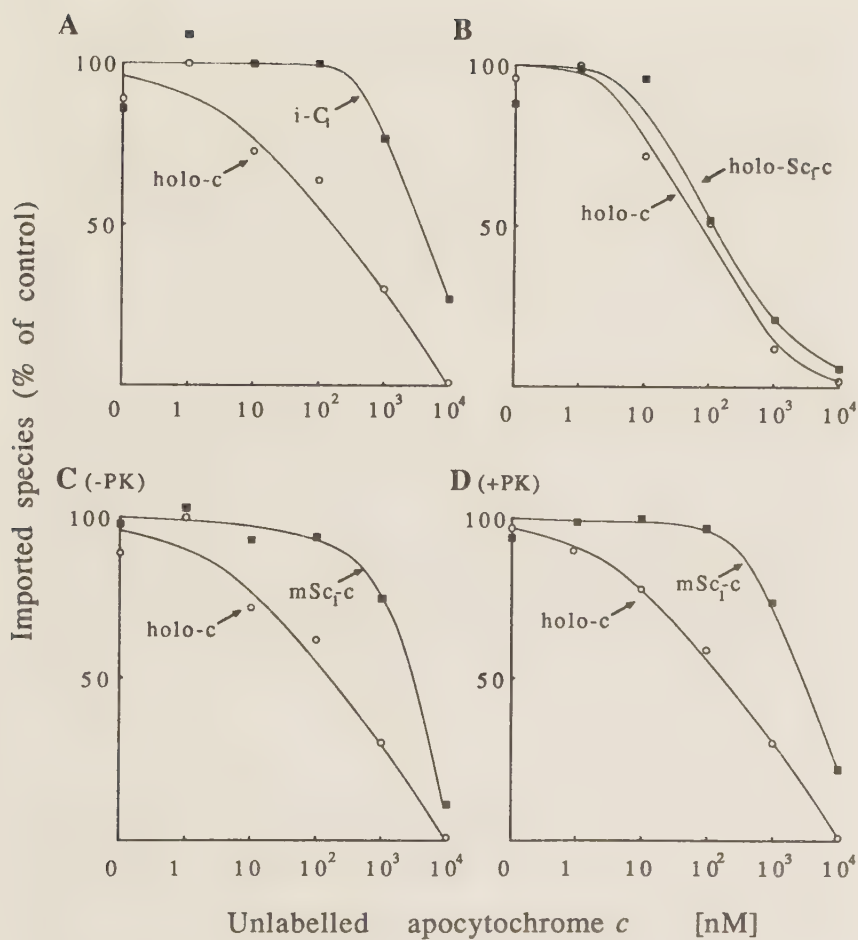
The precursors of Sc<sub>I</sub>-c, cytochrome c<sub>1</sub> and porin were synthesized in reticulocyte lysate in the presence of [<sup>35</sup>S]methionine. The various lysates (20 µl each) were incubated with isolated mitochondria (10 µg) and 50 µl water-soluble porin (dissolved in 0.1 M NaPi, pH 6.8, Pfaller and Neupert, 1987) at concentrations indicated. The final volume was adjusted to 200 µl with BSA-containing buffer. Samples were incubated for 40 min at 0 °C after which antimycin A, oligomycin and valinomycin were added to final concentrations of 8 µM, 20 µM, and 0.5 µM, respectively. Mitochondria were recovered by centrifugation and resuspended in 100 µl of BSA-containing buffer and incubated for a further 15 min at 25 °C. Samples were then cooled to 0 °C and in the case of porin were treated with 200 µg proteinase K/ml, whereas those for pSc<sub>I</sub>-c and cytochrome c<sub>1</sub> were treated with 20 µg proteinase K/ml. All samples were incubated for 30 min at 0 °C. Protease activity was halted by the addition of 1 ml of SEM buffer containing 2 mM PMSF. Mitochondria were reisolated by centrifugation and lysed in a SDS-containing buffer and all samples were resolved by SDS/PAGE. Imported porin (□), iC<sub>1</sub> (○) and mSc<sub>I</sub>-c (▲) were quantified by subsequent laser densitometry of fluorographs.

deficient mutant (which are unable to bind apocytochrome *c* or produce holocytochrome *c*) were examined for their ability to import pSc<sub>1</sub>-*c*.

In the first approach, unlabelled apocytochrome *c* was tested for its ability to compete for the import of pSc<sub>1</sub>-*c* into mitochondria (Figure 16). As a control, [<sup>35</sup>S]-labelled apocytochrome *c* and precytochrome *c*<sub>1</sub> were incubated with isolated mitochondria (separately) in the presence of varying concentrations of unlabelled apocytochrome *c* (Figure 16A). Holocytochrome *c* formation (from [<sup>35</sup>S]-apocytochrome *c*) was competed by the added apocytochrome *c*; half-maximal competition was observed at an apocytochrome *c* concentration of about 100 nM. Import of cytochrome *c*<sub>1</sub> and processing to iC<sub>1</sub>, however, was not competed by similar concentrations of apocytochrome *c*. Significantly (100-fold) higher levels of unlabelled apocytochrome *c* were required to observe reduced iC<sub>1</sub> accumulation. Unlabelled apocytochrome *c* could not specifically compete for the ΔΨ-dependent import of pSc<sub>1</sub>-*c* and processing to mSc<sub>1</sub>-*c* in either untreated (Figure 16C) or proteinase K-treated (Figure 16D) mitochondria.

This observation could be accounted for if the Sc<sub>1</sub>-*c* precursor has a higher affinity for the cytochrome *c* import pathway than apocytochrome *c*. Import of pSc<sub>1</sub>-*c* along the cytochrome *c* route (as measured by holo-pSc<sub>1</sub>-*c* formation) is, however, specifically competed for by apocytochrome *c* (Figure 16B) indicating that pSc<sub>1</sub>-*c* has about the same affinity as the legitimate apocytochrome *c* molecule.

In the second approach we employed the *cyt2-1* mutant of *Neurospora crassa*, the mitochondria of which are unable to bind apocytochrome *c* and are deficient in CCHL activity (Nargang *et al.*, 1988). A cytochrome *c* import pathway does not exist in the *cyt2-1* mitochondria. Import of pSc<sub>1</sub>-*c* into the matrix of *cyt2-1* mitochondria occurred with a similar efficiency as its import into wild-type mitochondria (not shown). Similarly pSc<sub>1</sub>-*c* import into the matrix of protease-treated *cyt2-1* mitochondria (Figure 17A, lanes 5-7) was comparable to its import into untreated mitochondria (Figure 17A, lanes 1-3). Import of pSc<sub>1</sub>-*c* into *cyt2-1* mitochondria along the cytochrome *c* pathway was, however, severely blocked (Figure 17A, lanes 4 and 8). Therefore, in the absence



**Figure 16. Apocytochrome *c* does not compete for the import of pSc<sub>J</sub>-*c* into the mitochondrial matrix**

[<sup>35</sup>S]-Labelled apocytochrome *c*, cytochrome *c*<sub>1</sub> and pSc<sub>J</sub>-*c* were imported into mitochondria under various conditions in the presence of varying concentrations of unlabelled apocytochrome *c* (at concentrations indicated) as described in Methods. *A. control import.* [<sup>35</sup>S]-Apocytochrome *c* was incubated with mitochondria in the presence of varying concentrations of unlabelled apocytochrome *c* for 20 min at 25 °C in the presence of NADH and then holocytochrome *c* formed was determined (○). [<sup>35</sup>S]-labelled precytochrome *c*<sub>1</sub> was imported into mitochondria in parallel incubation mixtures for 20 min at 25 °C in the presence of 2.5 mM MgCl<sub>2</sub> but in the absence of NADH. Protease-resistant intermediate-size cytochrome *c*<sub>1</sub> (iC<sub>1</sub>) was then determined (■). *B. Import along cytochrome *c* pathway.* Import of [<sup>35</sup>S]-apocytochrome *c* (○) and [<sup>35</sup>S]-pSc<sub>J</sub>-*c* (■) along the cytochrome *c* pathway was monitored by formation of the respective holo-forms as described in Methods. Import mixtures, containing antimycin A, oligomycin, valinomycin and 5 mM NADH, were incubated for 20 min at 25 °C before being immunoprecipitated for HPLC analysis of the resulting tryptic peptides. *C and D. Import of pSc<sub>J</sub>-*c* into the mitochondrial matrix of untreated and proteinase K treated mitochondria.* Untreated mitochondria (C) or proteinase K treated mitochondria (D) were incubated with [<sup>35</sup>S]-apocytochrome *c* (○) or [<sup>35</sup>S]-pSc<sub>J</sub>-*c* (■) for 20 min at 25 °C. Incubation mixtures containing [<sup>35</sup>S]-pSc<sub>J</sub>-*c* also contained 2.5 mM MgCl<sub>2</sub>. Import into the matrix was determined by formation of protease-resistant mature-size Sc<sub>J</sub>-*c* (mSc<sub>J</sub>-*c*). Holocytochrome *c* formation was determined by immunoprecipitation followed by HPLC analysis of the resulting tryptic peptides.

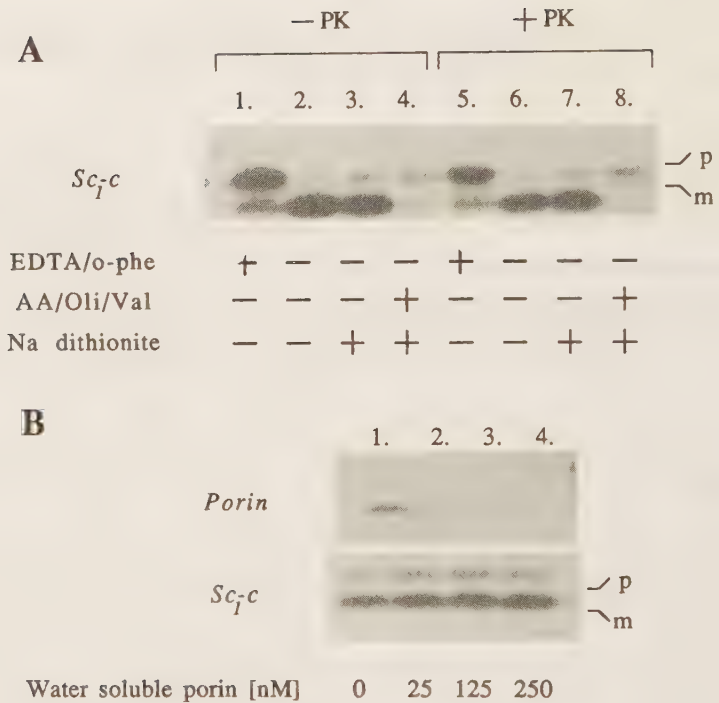
of both the cytochrome  $c_1$  receptor and a functional cytochrome  $c$  import apparatus, pSc $_I$ - $c$  was still able to enter the mitochondrial matrix with high efficiency.

Like in wild-type mitochondria, ws porin successfully blocked import at the level of GIP in isolated *cyt2-1* mitochondria, as judged by the inhibition of import of labelled porin (Figure 17B, upper panel). The *cyt2-1* mitochondria could still import pSc $_I$ - $c$  into the matrix, where it was processed to mSc $_I$ - $c$ , even when GIP was occupied by ws porin (Figure 17B, lower panel). Clearly the import of pSc $_I$ - $c$  into the mitochondrial matrix was not assisted by components of the cytochrome  $c$  import pathway.

#### 4.19 Functional intersection of the cytochrome $c$ - and matrix-import routes

We next asked if there was any overlap between the two pathways by initiating pSc $_I$ - $c$  import on the cytochrome  $c$  pathway and then to attempt to divert it onto a route leading to the matrix.

Mitochondria were treated with proteinase K to remove the cytochrome  $c_1$  receptor, and with antimycin A plus oligomycin to inhibit the formation of a membrane potential (Figure 18). Under these conditions pSc $_I$ - $c$  was bound to mitochondria without any further import along either the cytochrome  $c$  pathway (because of the absence of NADH or sodium dithionite) or the matrix pathway (because of the absence of  $\Delta\Psi$ ). The mitochondria containing the bound pSc $_I$ - $c$  were washed and reisolated, then pSc $_I$ - $c$  was chased along the cytochrome  $c$  pathway by the addition of a reductant (Figure 18A) or the matrix pathway by restoring the membrane potential (Figure 18B). Upon the addition of sodium dithionite, bound pSc $_I$ - $c$  was converted to holo-pSc $_I$ - $c$  with high efficiency (Figure 18A, tracks 1 vs 2). If KCl (0.3 M) was added following the binding incubation, but prior to the chase, subsequent formation of holo-pSc $_I$ - $c$  during the chase was unaffected (Figure 18A, track 3). This indicates that about 70 % of the bound pSc $_I$ - $c$  was initially present in a salt-resistant complex with CCHL and had entered a cytochrome  $c$  pathway; such a complex with CCHL has been described in detail for apocytochrome  $c$  by Nicholson *et al.*, 1988. On the other hand, over 50 % of the bound pSc $_I$ - $c$  could also be chased into the mitochondrial matrix, where it was processed to mSc $_I$ - $c$  (Figure 18B,



**Figure 17. Import of p*Sc<sub>I</sub>-c* into *cyt2-1* mitochondria**

A. Isolated *cyt2-1* mitochondria were either untreated (lanes 1-4) or treated with proteinase K (lanes 5-8) at a concentration of 50  $\mu\text{g/ml}$  as outlined in Methods. Import of [ $^{35}\text{S}$ ]-p*Sc<sub>I</sub>-c* was performed exactly as described in the legend to Figure 13, except that the mutant *cyt2-1* mitochondria were used instead of the wild-type mitochondria.

B. [ $^{35}\text{S}$ ]-p*Sc<sub>I</sub>-c* and porin were imported into *cyt2-1* mitochondria in the presence of increasing concentrations of water-soluble porin, essentially as described in the legend to Figure 15. Following the chase reaction for 15 min at 25  $^{\circ}\text{C}$ , import of porin was assessed by protease resistance following treatment with 200  $\mu\text{g}$  proteinase K/ml. Import of p*Sc<sub>I</sub>-c* into the matrix was monitored by m*Sc<sub>I</sub>-c* formation. Mitochondria were lysed in a SDS-containing buffer and all samples were resolved by SDS/PAGE. The bands corresponding to protease-resistant porin and to *Sc<sub>I</sub>-c* in the resulting films are shown.

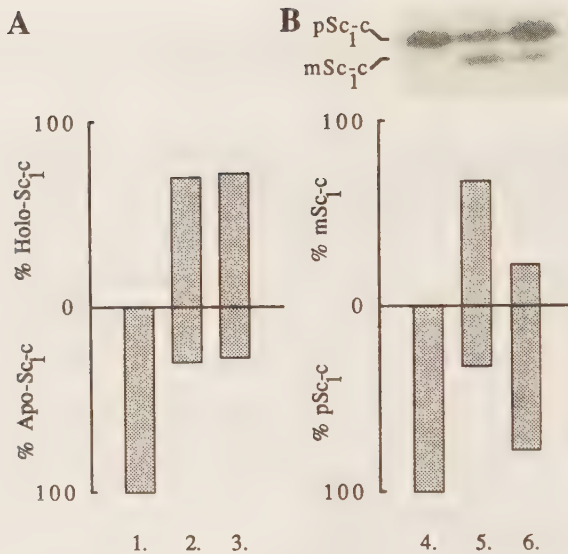
track 4 vs 5). This route was severely impeded by the presence of salt (Figure 18B, track 6).

In summary, these findings indicate that pSc<sub>1</sub>-c bound to mitochondria existed in an equilibrium from which it could be chased along either one of two distinct import pathways. The majority of the bound pSc<sub>1</sub>-c had entered the cytochrome *c* import way, but a large proportion of it could be re-routed to the matrix upon re-establishing the membrane potential. It may be concluded, therefore, that initiation of a precursor along one mitochondrial import pathway (i.e. the cytochrome *c* route) does not necessarily commit it to that pathway. As this is indeed the case, it implies that a functional intersection or exchange between the cytochrome *c* and matrix import pathways can exist.

#### 4.20 Import of pLc<sub>1</sub>-c into mitochondria

In addition to pSc<sub>1</sub>-c, a second hybrid protein, pLc<sub>1</sub>-c, which is comprised of the entire cytochrome *c*<sub>1</sub> presequence joined to the amino terminus of apocytochrome *c* was constructed (Figure 8B). Like pSc<sub>1</sub>-c, pLc<sub>1</sub>-c also contains dual targeting information while at the same time, membrane insertion activity. However, in addition to the matrix-targeting domain of the cytochrome *c*<sub>1</sub> presequence which pSc<sub>1</sub>-c contains, pLc<sub>1</sub>-c also contains the second, hydrophobic, domain of the cytochrome *c*<sub>1</sub> presequence. As mentioned previously, this second domain of the presequence is thought to direct the export of the intermediate-size cytochrome *c*<sub>1</sub> (iC<sub>1</sub>) from the mitochondrial matrix across the inner membrane to its final location exposed to the intermembrane space (Hartl *et al.*, 1987). Hence the import pathways of this fusion protein, pLc<sub>1</sub>-c have also been characterized. The similarities and differences of the import pathways of pLc<sub>1</sub>-c, compared to those of the pSc<sub>1</sub>-c fusion protein will be discussed with reference to their relevance on mitochondrial targeting information and on the role of receptors and GIP in mediating the translocation of mitochondrial precursor proteins.

The precursor of the Lc<sub>1</sub>-c fusion protein (pLc<sub>1</sub>-c) was synthesized in the presence of [<sup>35</sup>S]cysteine in a rabbit reticulocyte lysate and was imported to a protease-resistant location in mitochondria at 25 °C under various incubation conditions (Figure 19).



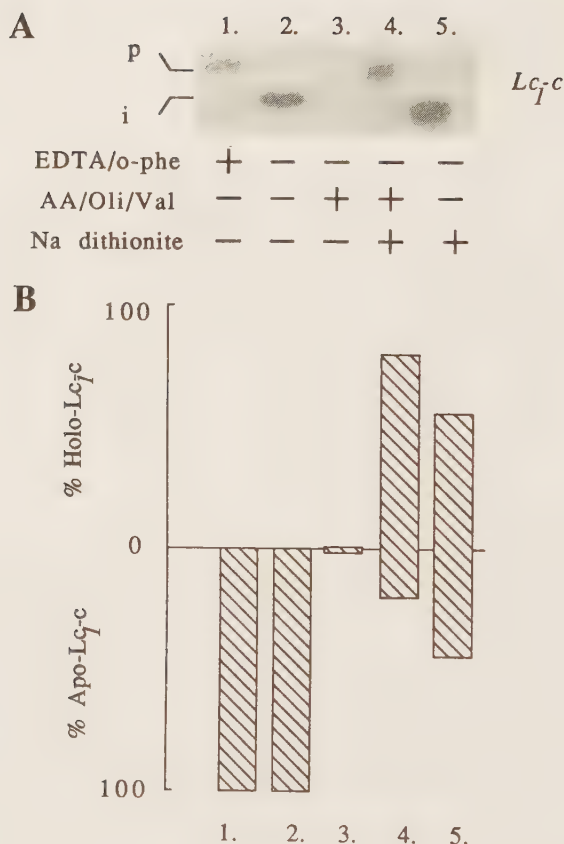
**Figure 18. pScI-c bound to protease-treated mitochondria can be imported by either matrix- or cytochrome c-pathways**

[<sup>35</sup>S]Cysteine-labelled pScI-c (75  $\mu$ l reticulocyte lysate) was bound to protease-treated mitochondria (300  $\mu$ g protein, in a final volume of 600  $\mu$ l in SME buffer) in the presence of holocytochrome c (horse heart), antimycin A and oligomycin for 10 min at 0 °C. The mitochondria were reisolated by centrifugation and resuspended in fresh SEM buffer. The bound pScI-c was chased either along the cytochrome c- or matrix-pathway in mixtures equivalent to 50  $\mu$ g of mitochondrial protein (containing bound pScI-c) in a final volume of 100  $\mu$ l in BSA buffer. **A. Chase along the cytochrome c pathway.** Samples were incubated for 20 min at 25 °C either in the presence of antimycin A, oligomycin and valinomycin (tracks 1, 2 and 3), sodium dithionite (1 mg/ml) (tracks 2 and 3) or in the presence of KCl (0.3 M final concentration). Total cytochrome c was immunoprecipitated from the samples and the amount of apo- and holo-pScI-c was determined by HPLC analysis of tryptic peptides as described in Methods. Results are given as the percentage of the total mitochondrial-associated cytochrome c present as either apo-ScI-c or holo-ScI-c. **B. Chase along the matrix-import pathway.** pScI-c was chased to mature-size ScI-c (mScI-c) by incubation for 20 min at 25 °C either in the presence of antimycin A, oligomycin and valinomycin (track 4) or in the presence of 0.2 mM N,N,N',N'-tetramethylphenylenediamine plus 8 mM K-ascorbate (from a stock adjusted to pH 7.2 with KOH) to re-establish a membrane potential (tracks 5 and 6) or in the presence of 0.3 M KCl (track 6). All samples contained 2.5 mM MgCl<sub>2</sub> to activate the matrix processing peptidase. Following incubation, mitochondria were reisolated, lysed in SDS-containing buffer and resolved by SDS/PAGE. The ScI-c bands from a fluorograph of the resulting gel are shown. The bands were quantified by laser densitometry and the percentage precursor (% pScI-c) and processed mature-size (% mScI-c) are given.

Following protease treatment, samples were divided and analysed either by HPLC analysis, to allow quantification of apo- and holocytochrome *c*, or by SDS/PAGE and fluorography in order to determine the levels of pLc<sub>1</sub>-*c* and processed intermediate-size Lc<sub>1</sub>-*c* (iLc<sub>1</sub>-*c*).

In the first import reaction, where EDTA and *o*-phenanthroline were present (Figure 19A, lane 1), accumulation of protease-resistant pLc<sub>1</sub>-*c* was observed. When import was performed in the presence of MgCl<sub>2</sub>, pLc<sub>1</sub>-*c* was imported and converted to iLc<sub>1</sub>-*c* (Figure 19A, lane 2). Import of pLc<sub>1</sub>-*c* into mitochondria did not occur in the absence of a membrane potential (Figure 19A, lane 3), unless a reductant such as sodium dithionite was present to power holocytochrome *c* formation and consequently import along a cytochrome *c* pathway (Figure 19A, lane 4). Under these conditions, heme-containing pLc<sub>1</sub>-*c* was accumulated in a protease-resistant location (Figure 19A, B, lane and track 4). Formation of holo-Lc<sub>1</sub>-*c* did not occur unless a reductant was included in the incubation (Figure 19B, tracks 4 and 5 vs tracks 1, 2 and 3). When both the cytochrome *c*<sub>1</sub> and cytochrome *c* pathways were active (i.e. + $\Delta\Psi$ , + reductant) the only protease-resistant species to accumulate was iLc<sub>1</sub>-*c* (Figure 19A, lane 5). Formation of holo-iLc<sub>1</sub>-*c* was almost as efficient as that of holo-pLc<sub>1</sub>-*c* formation (Figure 19B, track 5 vs 4), thus suggesting that, in contrast to holo-Sc<sub>1</sub>-*c* formation, holo-Lc<sub>1</sub>-*c* formation was entirely independent of  $\Delta\Psi$ , a situation similar to the formation of the authentic holocytochrome *c* (Figure 9, lanes 5, 6). Thus it would appear from these results that the holo-Lc<sub>1</sub>-*c* may accumulate in the mitochondria either as a precursor- or intermediate-size Lc<sub>1</sub>-*c* depending whether a membrane potential was present or not.

We conclude therefore, that both the matrix- and cytochrome *c*-targeting information in the pLc<sub>1</sub>-*c* hybrid protein are functional and thus pLc<sub>1</sub>-*c* can be selectively imported into mitochondria along either pathway.



**Figure 19. Import of p*LcI-c* into mitochondria**

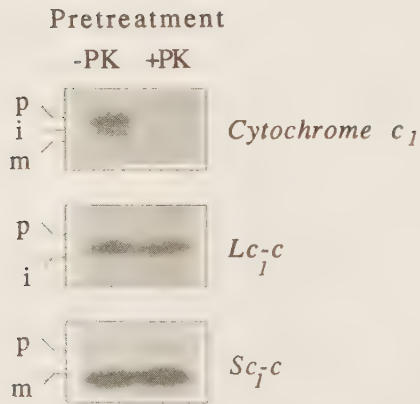
The precursor of *LcI-c*, synthesized in the presence of [ $^{35}$ S]cysteine, was imported into isolated mitochondria (100  $\mu$ g protein) at 25 °C in the presence of 5 mM EDTA plus 0.2 mM *o*-phenanthroline (track 1), 2.5 mM  $\text{MgCl}_2$  (tracks 2, 3, 4 and 5), sodium dithionite (1 mg/ml) (track 4 and 5) or a mixture of antimycin (AA), oligomycin (Oli) and valinomycin (val) (tracks 3 and 4) as described in Methods. Following protease treatment, samples were divided in half and *LcI-c* was immunoprecipitated. (A) One set of samples were analysed by SDS/PAGE and fluorography. The bands corresponding to *LcI-c* in the resulting film are shown. (B) The other set of samples were dissociated with urea, digested with trypsin and subjected to reverse-phase HPLC. The amounts of apo- and holo-*LcI-c* were quantified by collecting the corresponding peptides and determining their radioactivity. Results are given as the percentage of the total mitochondrial-associated cytochrome *c* present as either apo-*LcI-c* or holo-*LcI-c*.

#### 4.21 Analysis of the requirement of the cytochrome $c_1$ receptor and GIP for the import of pLc<sub>1</sub>-c

Mitochondria were pretreated with proteinase K (50 µg/ml) and were tested for their ability to import cytochrome  $c_1$ , pSc<sub>1</sub>-c and pLc<sub>1</sub>-c in comparison to untreated mitochondria (Figure 20). Import into mitochondria was controlled by the resistance to externally added proteinase K following the import incubation. The import of cytochrome  $c_1$  was abolished in the protease-treated mitochondria, thus demonstrating clearly that the treatment of mitochondria with the protease had successively removed the cytochrome  $c_1$  receptor. The accumulation of protease-resistant iLc<sub>1</sub>-c, like the accumulation of mSc<sub>1</sub>-c, was also independent on the presence on the cytochrome  $c_1$  receptor.

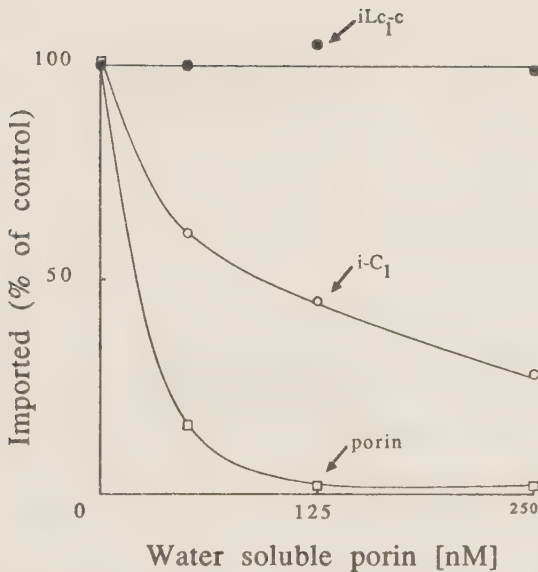
The question as to whether pLc<sub>1</sub>-c import into the matrix was dependent on GIP was then addressed. Ws porin was prepared and tested for its ability to compete against pLc<sub>1</sub>-c import into the matrix at the level of the GIP. As shown in Figure 21, ws porin was unable to inhibit pLc<sub>1</sub>-c import at all concentrations used, thus the membrane insertion activity of the cytochrome  $c$  domain renders the function of GIP indispensable.

In summary, despite the interruption of the two membrane active domains (i.e. the first part of the cytochrome  $c_1$  presequence and of the apocytochrome  $c$  part) with the second part of the cytochrome  $c_1$  presequence, the presence of the cytochrome  $c$  domain as part of the pLc<sub>1</sub>-c fusion protein endows it with the properties of receptor independence. This second domain of the presequence is very hydrophobic and therefore, it may also become inserted into the outer membrane along with the apocytochrome  $c$  and thus does not hinder the matrix-targeting domain from being delivered to the vicinity of the  $\Delta\Psi$ -dependent step of matrix import. Thus it would be interesting to investigate whether by exchanging this hydrophobic sequence for a hydrophilic one, whether the matrix-import of such a fusion protein would be receptor dependent.



**Figure 20.** Import of pLc<sub>I</sub>-c into the matrix is not dependent on the cytochrome c<sub>1</sub> receptor

pLc<sub>I</sub>-c was tested for its ability to be imported into protease-treated mitochondria: proteinase K pretreatment of mitochondria was carried out as described in Methods. Protease-treated mitochondria (+PK) and control mitochondria (-PK) were tested for their ability to import [<sup>35</sup>S]-labelled precytochrome c<sub>1</sub>, pSc<sub>I</sub>-c and pLc<sub>I</sub>-c in the presence of 2.5 mM MgCl<sub>2</sub> for 20 min at 25 °C. Import into the matrix was determined by the formation of protease-resistant intermediate-size cytochrome c<sub>1</sub> (iC<sub>1</sub>) and Lc<sub>I</sub>-c (iLc<sub>I</sub>-c) and mature-size Sc<sub>I</sub>-c (mSc<sub>I</sub>-c). All samples were resolved by SDS/PAGE and the corresponding bands from the resulting fluorographs are depicted.



**Figure 21 Import of pLc<sub>1</sub>-c into the matrix does not require GIP**

The precursors of Lc<sub>1</sub>-c, cytochrome *c*<sub>1</sub> and porin, synthesized in reticulocyte lysate in the presence of [<sup>35</sup>S]methionine, were imported into mitochondria in the presence of varying concentrations of unlabelled water-soluble porin (at concentrations indicated) as described in Figure 15. Following the incubation period, import of porin was controlled by protease-resistance following treatment with 200 µg proteinase K/ml, while import of cytochrome *c*<sub>1</sub> and pLc<sub>1</sub>-c was controlled by resistance to 20 µg proteinase K/ml. Reisolated mitochondria were then lysed with SDS-sample buffer and all samples were resolved by SDS/PAGE. Imported porin (□), iC<sub>1</sub> (○) and iLc<sub>1</sub>-c (●) were quantified by subsequent laser densitometry of the fluorographs of the resulting gels.

#### 4.22 Import of pLc<sub>I</sub>-c via translocation contact sites

Transport of precursors across the two mitochondrial membranes occurs in a single step at contact sites between the outer and inner membranes (Schleyer & Neupert, 1985; Schwaiger *et al.*, 1987). Precursor proteins can be trapped and analysed in these contact sites (translocation intermediates) by various different approaches such as transport at low temperature or transport after the binding of precursor proteins to antibodies. These partially transported precursors are accessible to externally added proteases yet have undergone processing by the matrix-located processing peptidase. The latter approach, namely of prebinding antibodies to the Lc<sub>I</sub>-c precursor protein, was performed in order to investigate whether import of pLc<sub>I</sub>-c into the matrix occurred via contact sites.

[<sup>35</sup>S]Methionine-labelled pLc<sub>I</sub>-c was synthesized in reticulocyte lysate and was incubated with control serum or with antisera against apocytochrome *c*. Isolated mitochondria were combined with the reticulocyte lysates and transfer was performed under different conditions. Following incubation, Lc<sub>I</sub>-c was immunoprecipitated from the samples which received control serum following the addition of cytochrome *c* antisera, however, immunoprecipitation from mitochondria of those incubations that initially had received specific antisera, received no further antisera. All immunocomplexes were purified with protein A-Sepharose and analysed (Figure 22). Processing of pLc<sub>I</sub>-c to iLc<sub>I</sub>-c was dependent on a membrane potential and it occurred irrespective of the presence or absence of prebound specific antibodies (Figure 22, lanes 1 and 2). The antibody-prebound iLc<sub>I</sub>-c, however, remained sensitive to added protease, unlike the pLc<sub>I</sub>-c which was imported in the presence of control serum. The integrity of the mitochondrial outer membrane was not adversely affected by these incubation treatments (results not shown). Thus the antibody-Lc<sub>I</sub>-c complex was not completely translocated into mitochondria, however, it was processed to the intermediate-size form.

It may thus be concluded that Lc<sub>I</sub>-c could form a translocation intermediate, spanning both outer and inner mitochondrial membranes, namely, at contact sites.

### A. Control serum



### B. Anti-cyt.c serum



**Figure 22. Precursor of  $Lc_7c$  bound to antibodies is processed by mitochondria**  
 Two aliquots (each 350  $\mu$ l) of reticulocyte lysate (50  $\mu$ l), containing [ $^{35}$ S]methionine-labeled  $Lc_7c$  precursor protein, supplemented with BSA-buffer (see Methods) were incubated either with 40  $\mu$ l control antiserum (A) or 40  $\mu$ l anti-apocytochrome  $c$  serum, for 30 min at 25  $^{\circ}$ C. The mixes were then divided into 4 aliquots (each 90  $\mu$ l) and were incubated with freshly isolated mitochondria (20  $\mu$ g) either in the presence of antimycin A, oligomycin, valinomycin (lane 2) or not (lane 1, 3 and 4) and were incubated for 20 min at 25  $^{\circ}$ C. Samples were cooled to 0  $^{\circ}$ C following incubation and samples 3 and 4 only were treated with 30  $\mu$ g proteinase K/ml for 30 min at 0  $^{\circ}$ C. Protease activity was halted by the addition of 1 ml SEM containing 2 mM PMSF and mitochondria were reisolated. Mitochondria of samples 1 to 3 were lysed in Triton X-100 containing buffer (see Methods) and those of reaction 4 in an 1 % SDS, 10 mM Tris-HCl, pH 8.0 followed by boiling at 95  $^{\circ}$ C for 5 min and then were also diluted with 1 ml of Triton X-100 containing buffer. After a clarifying spin, anti-cytochrome  $c$  serum and protein A-Sepharose were added to control samples (A). To the other samples (B), only protein A-Sepharose was added. Immunoprecipitation and washing of immunocomplexes were performed as described in Methods. Immunocomplexes were dissociated in an SDS-containing buffer and were analysed by SDS/PAGE. The corresponding bands from the resulting fluorographs are depicted.

### 4.23 Functional dissection of the import pathway of pLc<sub>I</sub>-c

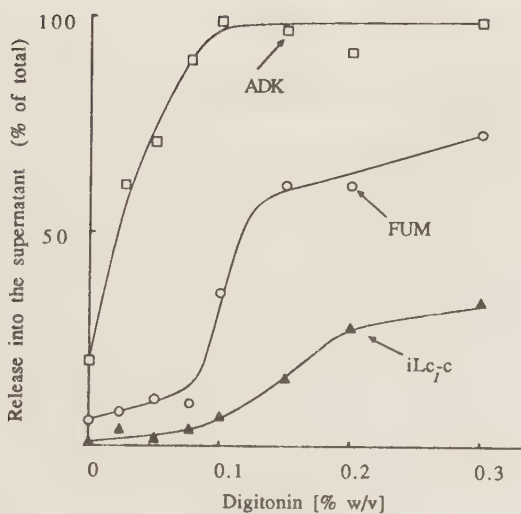
It has thus been demonstrated that both sets of targeting information present in pLc<sub>I</sub>-c are active and can serve to selectively target pLc<sub>I</sub>-c either along a cytochrome *c*- or matrix-pathway (Figure 19). It also appeared from these results that the holo-Lc<sub>I</sub>-c may be accumulated in the mitochondria either as a precursor- or intermediate-size Lc<sub>I</sub>-c depending on whether a membrane potential was present or not. Therefore, in the presence of both  $\Delta\Psi$  and a reductant, pLc<sub>I</sub>-c underwent two maturation events upon import into mitochondria, namely, cleavage by the matrix processing peptidase and the covalent attachment of heme. The question thus arises as to the chronological sequence of these events. Is pLc<sub>I</sub>-c, like precytochrome *c*<sub>1</sub>, initially imported into the matrix where it is processed to its intermediate-size, iLc<sub>I</sub>-c, and then is retranslocated across the inner membrane after which it receives the heme, a reaction that could be catalysed either by the cytochrome *c*<sub>1</sub> heme lyase (CC<sub>1</sub>HL) or by cytochrome *c* heme lyase (CCHL)? Alternatively, the following sequence of events could be occurring, pLc<sub>I</sub>-c initially interacts with CCHL, resulting in holo-pLc<sub>I</sub>-c formation and thus triggering translocation across the outer membrane, in doing so the matrix-targeting sequence may gain access to the  $\Delta\Psi$ -dependent step and as a result translocation (at least of the presequence) across the inner membrane occurs. Consequently cleavage by the matrix-located processing peptidase (MPP) takes place, resulting in the formation of holo-iLc<sub>I</sub>-c. A functional dissection of the import pathway of pLc<sub>I</sub>-c was performed in order to elucidate which of these two possibilities was in fact correct.

### 4.24 Localization of the apo-iLc<sub>I</sub>-c accumulated in the mitochondria

As previously reported the precursor of the Lc<sub>I</sub>-c fusion protein (pLc<sub>I</sub>-c) could be imported into the matrix and be processed to iLc<sub>I</sub>-c in the presence of the a membrane potential and divalent cations. In order to determine the localization of the iLc<sub>I</sub>-c species, import of [<sup>35</sup>S]methionine-labelled pLc<sub>I</sub>-c into the mitochondria was performed at 25 °C in the presence of MgCl<sub>2</sub>. Mitochondria were reisolated and treated with increasing concentrations of digitonin to sequentially open the intermembrane space and matrix compartments of the mitochondria. Prior to the separation into pellet and

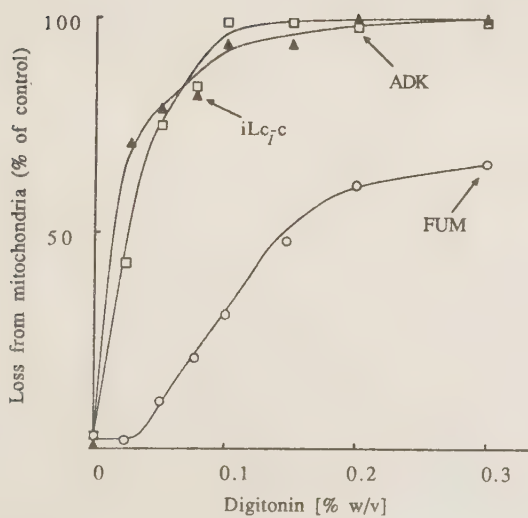
A

-PK



B

+PK



**Figure 23. Submitochondrial localization of imported apo-iLc<sub>J</sub>-c by digitonin treatment of mitochondria**

The [<sup>35</sup>S]methionine-labelled pLc<sub>J</sub>-c was imported into mitochondria (1.5 mg protein, 2 ml final volume) at 25 °C in the presence of 2.5 mM MgCl<sub>2</sub> for 15 min. Following a two-fold dilution, mitochondria were reisolated and resuspended in SEMK-buffer containing 100 mM KCl (SEMK) and divided into eight aliquots. Digitonin was added to concentrations of 0 to 0.3 % (w/v) and incubated for 2 min at 0 °C in a final volume of 40 µl and at a protein concentration of 5 mg/ml. Afterwards samples were diluted 20-fold with SEMK and prior to separation into pellet and supernatant fractions, samples were divided and one half was treated with 25 µg proteinase K/ml for 30 min at 0 °C. Protease activity was then halted by the addition of PMSF (2 mM) and samples were separated into pellet and supernatant fractions by centrifugation for 10 min at 27,000 x g (Beckman, JA-20 rotor). Supernatants of the non protease-treated samples were adjusted to 1 % (w/v) Genapol and pellets of all samples were resuspended in SEMK plus 1 % (w/v) Genapol, to the same volume as the supernatants. Both the pellet and supernatant fractions were divided in half. Lc<sub>J</sub>-c was immunoprecipitated (see Methods) from one set of samples and analysed by SDS/PAGE, where the resulting fluorographs were quantified by laser densitometry (panel A and B, ▲ ). The remaining parts of the samples were used for determination of marker enzyme activities, adenylate kinase ( □ and fumarase ○ ). For panel A, the activities recovered in the pellet and supernatant fractions were set to 100 % for each digitonin concentration, however, for panel B activity present in the first sample which received no digitonin was set as the total 100 %. The loss from the mitochondria is plotted. ADK, adenylate kinase; Fum, fumarase.

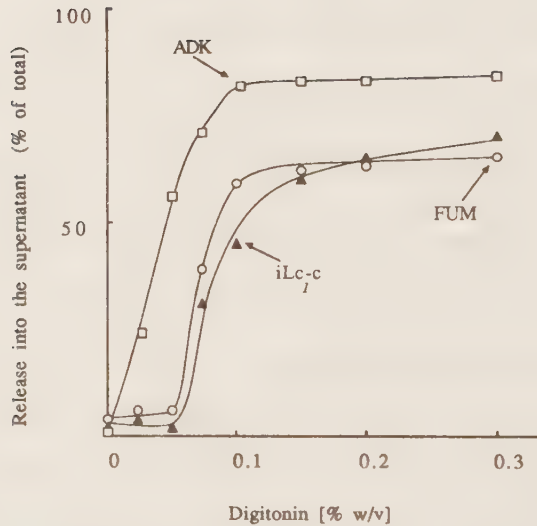
supernatant fractions, samples were divided and one half of each sample was treated with proteinase K.

iLc<sub>1</sub>-c accumulated at 25 °C remained membrane bound (Figure 23A), as only low levels of iLc<sub>1</sub>-c were released from the membrane fraction into the supernatant upon digitonin treatment. Measurement of adenylate kinase and fumarase activities, though, indicated that nearly complete loss of the soluble components of the intermembrane space and matrix compartments was achieved at the highest levels of digitonin. The largest proportion of iLc<sub>1</sub>-c, thus, fractionated with the membrane pellet fraction. If the digitonin treatment was followed by a proteinase K treatment (Figure 23B), however, imported iLc<sub>1</sub>-c remained protected against added protease only up to the point where the intermembrane space was opened as a result of the digitonin treatment. This disruption of the outer membrane by the digitonin was monitored by the release of the intermembrane space marker, adenylate kinase.

It was concluded, therefore, from these data, that the imported iLc<sub>1</sub>-c was exposed to the intermembrane space while being anchored to the inner mitochondrial membrane, presumably via the remaining, hydrophobic core of the cytochrome *c*<sub>1</sub> presequence. The topology of the imported iLc<sub>1</sub>-c resembled that of the intermediate-size cytochrome *c*<sub>1</sub> accumulated at 25 °C in the absence of reductants (Hartl *et al.*, 1987; Nicholson *et al.*, 1989).

#### **4.25 Accumulation of the intermediate-size Lc<sub>1</sub>-c (iLc<sub>1</sub>-c) in the mitochondrial matrix**

The precursor of the Lc<sub>1</sub>-c fusion protein was synthesized in the presence of [<sup>35</sup>S]methionine in a reticulocyte lysate. Import of the precursor into isolated mitochondria was performed in the presence of MgCl<sub>2</sub> at 8 °C. Following protease treatment, mitochondria were reisolated and treated with increasing concentrations of digitonin to sequentially release the contents of the intermembrane space and matrix compartments. Accumulated iLc<sub>1</sub>-c was released from the mitochondria upon a digitonin treatment that correlated with the release of the matrix marker, fumarase (Figure 24).



**Figure 24 .  $iLcJ-c$  can be accumulated as a soluble species in the matrix**

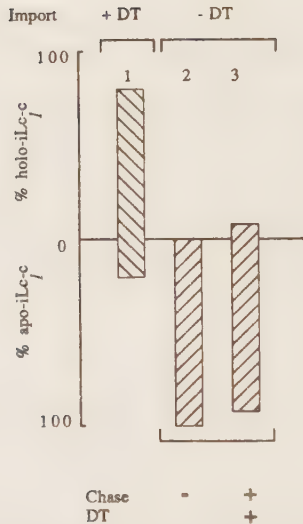
The [ $^{35}$ S]methionine-labelled  $pLcJ-c$  (200  $\mu$ l reticulocyte lysate) was imported into freshly isolated mitochondria (0.5 mg protein, 1 ml final volume) at 8  $^{\circ}$ C in the presence of 2.5 mM  $MgCl_2$  for 30 min. The sample was cooled to 0  $^{\circ}$ C and treated with 20  $\mu$ g proteinase K/ml for 30 min at 0  $^{\circ}$ C. Protease activity was halted by the addition of 2 ml SEM containing 2 mM PMSF, and mitochondria were resuspended in SEM buffer containing 100 mM KCl (SEMK) and divided into 8 aliquots. Digitonin treatment was performed as described in Figure 23. Following dilution samples were separated into pellet and supernatant fractions by centrifugation for 10 min at 27,000  $\times$  g (Beckman, JA-20 rotor). Supernatants were adjusted to 1 % (w/v) Genapol and pellets were resuspended in SEMK plus 1 % (w/v) Genapol to the same volume as the supernatants. Both supernatant and pellet samples were divided in half.  $iLcJ-c$  was immunoprecipitated from one set of samples and analysed by SDS/PAGE and the resulting fluorographs were quantified by laser densitometry ( $iLcJ-c$ ,  $\blacktriangle$ ). The remaining parts of the samples were used for determination of marker enzyme activities. For each digitonin concentration, the activities recovered from the pellet and supernatant fractions were set at 100 %. The release from the mitochondria into the supernatant is plotted. ADK, adenylate kinase (  $\square$  ) FUM, fumarase (  $\circ$  ).

This results suggest that at low temperature (8 °C) imported  $iLc_I$ - $c$  can be accumulated in the mitochondrial matrix as a soluble species. It may also be concluded that  $pLc_I$ - $c$  is initially imported into the matrix where it is processed by the matrix processing peptidase, prior to retranslocation across the inner membrane to its final location, namely, exposed to the intermembrane space (see Figure 23). Lowering the temperature, thus prevents the retranslocation of  $iLc_I$ - $c$  and stalls the import of  $pLc_I$ - $c$  at the level of the matrix; in this respect the import of  $pLc_I$ - $c$  is very similar to the import pathway of cytochrome  $c_1$  (Hartl *et al.*, 1987).

#### **4.26 Conversion of accumulated apo- $iC_1$ and apo- $iLc_I$ - $c$ to their respective holo-forms**

Cytochrome  $c_1$  can be accumulated at 25 °C in mitochondria as an intermediate-size species ( $iC_1$ ) in the absence of heme. This apo- $iC_1$ , which is topologically exposed to the intermembrane space while being anchored to the inner membrane, forms a salt-resistant complex with cytochrome  $c_1$  heme lyase ( $CC_1HL$ ) (Nicholson *et al.*, 1989). Upon addition of a reductant, such as sodium dithionite this apo- $iC_1$  can be chased to its holo-form, followed by processing to mature-size cytochrome  $c_1$  ( $mC_1$ ). The question thus arises as to whether apo- $iLc_I$ - $c$  accumulated in the same mitochondrial sublocation, i.e. exposed to the intermembrane space could also be chased to its holo-counterpart by adding a reductant.

Thus the precursor to the fusion protein,  $pLc_I$ - $c$ , was synthesized in a reticulocyte lysate in the presence of [ $^{35}S$ ]cysteine. The lysate was incubated with isolated mitochondria either in the presence or absence of sodium dithionite. Following incubation, the samples were treated with proteinase K, to digest precursor that was not imported into the mitochondria, and then the mitochondria were reisolated by centrifugation. The sample which was incubated in the presence of sodium dithionite was kept on ice, while the remaining sample was divided into two aliquots, and incubated at 25 °C for 5 min either in the presence of sodium dithionite or not. All samples were then analysed by HPLC, to allow quantitation of apo- and holo- $iLc_I$ - $c$  levels (Figure 25).



**Figure 25. Following import, apo-iLc<sub>1</sub>-c cannot be chased to holo-iLc<sub>1</sub>-c.**

The Lc<sub>1</sub>-c precursor was synthesized in the presence of [<sup>35</sup>S]cysteine and was imported into isolated mitochondria either in the presence (track 1) or absence of sodium dithionite (track 2 and 3).

a). *Accumulation of protease-resistant holo-iLc<sub>1</sub>-c*: Mitochondria (50 µg protein) were incubated [<sup>35</sup>S]-labelled Lc<sub>1</sub>-c precursor at 25 °C in the presence of 2.5 mM MgCl<sub>2</sub>, 3 µM hemin and sodium dithionite (1 mg/ml) for 20 min (see Methods). The sample was cooled and treated with 20 µg proteinase K/ml for 30 min at 0 °C. The protease activity was then halted and mitochondria were reisolated by centrifugation and lysed in Triton X-100 containing buffer. Lc<sub>1</sub>-c was immunoprecipitated (see Methods) and following dissociation of the immunocomplex with urea, peptides generated by digestion with trypsin were resolved by reverse-phase HPLC. The amounts of protease-resistant apo- and holo-iLc<sub>1</sub>-c were quantified by collecting the corresponding peptides and determining their radioactivity.

b). *Accumulation of protease-resistant apo-iLc<sub>1</sub>-c and subsequent chase to holo-iLc<sub>1</sub>-c*: Mitochondria (150 µg protein) were incubated with [<sup>35</sup>S]-labelled Lc<sub>1</sub>-c precursor at 25 °C in the presence of 2.5 mM MgCl<sub>2</sub> for 20 min (see Methods). Proteinase K treatment was performed following the incubation as described above for (a) and mitochondria were reisolated and suspended at a protein concentration of 0.5 mg/ml in SEM buffer. Two aliquots (equivalent to 50 µg protein) were then further incubated for 5 min at 25 °C in the presence of 3 µM hemin (track 2 and 3) and 1 mg sodium dithionite/ml (track 3) (final volume 200 µl in SEM buffer). The samples were lysed in the Triton X-100 containing buffer and Lc<sub>1</sub>-c was immunoprecipitated. The amounts of apo-iLc<sub>1</sub>-c and holo-iLc<sub>1</sub>-c formed upon chase were determined as described above. All results are given as the percentage of the total protease-resistant iLc<sub>1</sub>-c present as either apo-iLc<sub>1</sub>-c or holo-iLc<sub>1</sub>-c. The intermediate-size Lc<sub>1</sub>-c was the only species to accumulate under both of these import conditions (results not shown).

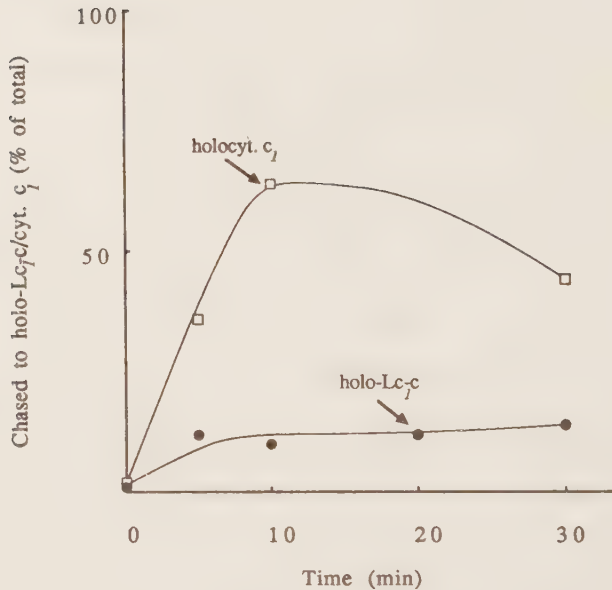
When import was carried out in the presence of sodium dithionite, almost all of the  $iLc_I-c$  which accumulated was holo in nature (Figure 25, track 1). The intermediate-size  $Lc_I-c$  was the only species to accumulate under these import conditions (result not shown). However, no holo-formation of the apo- $iLc_I-c$ , which had been accumulated in the absence of reductants, occurred in the subsequent chase incubation if reducing agents were omitted (Figure 25, track 2) or included (Figure 25, track 3).

That this failure of the apo- $iLc_I-c$  to be chased to holo- $iLc_I-c$  did not represent a kinetic problem is shown in Figure 26. The kinetics of holocytochrome  $c_1$  formation from accumulated apo-intermediate size  $c_1$  have been previously determined (Nicholson *et al.*, 1989). The precursors of cytochrome  $c_1$  and  $Lc_I-c$  were synthesized in reticulocyte lysate in the presence of [ $^{35}$ S]cysteine. Lysates were incubated (separately) with mitochondria in the absence of reductants at 25 °C for 20 min. Following incubation samples were protease-treated and the mitochondria were reisolated. Chase of the accumulated apo-intermediate-size cytochrome  $c_1$  and  $Lc_I-c$  was performed by the addition of sodium dithionite and incubation at 25 °C for the time periods indicated. Following the incubations, cytochrome  $c_1$  and  $Lc_I-c$  were immunoprecipitated and HPLC analysis was performed to quantify the levels of the apo- and holo-forms of both cytochrome  $c_1$  and  $Lc_I-c$  (Figure 26).

As previously demonstrated (Nicholson *et al.*, 1989), an incubation period of 10 min was optimal for holocytochrome  $c_1$  formation. Irrespective of the time period, however, no efficient holo- $iLc_I-c$  formation was observed. It may be concluded therefore that, once accumulated in mitochondria,  $iLc_I-c$  cannot be converted to holo- $iLc_I-c$ . These results would tend to suggest that the event of covalent heme attachment to  $pLc_I-c$  must occur prior to its entry and/or processing in the mitochondrial matrix.

#### 4.27 Substrate requirement of the cytochrome $c_1$ heme lyase

The covalent attachment of heme to apocytochrome  $c_1$  is catalysed by the enzyme cytochrome  $c_1$  heme lyase  $CC_1HL$  and occurs at the outer face of the inner membrane (Nicholson *et al.*, 1989).  $CC_1HL$  is clearly distinct from cytochrome  $c$  heme lyase



**Figure 26. Kinetics of holocytochrome  $c_1$  and holo-iLc $_J$ -c formation from the intermediate-size apocytochrome  $c_1$  and apo-iLc $_J$ -c accumulated *in vitro***

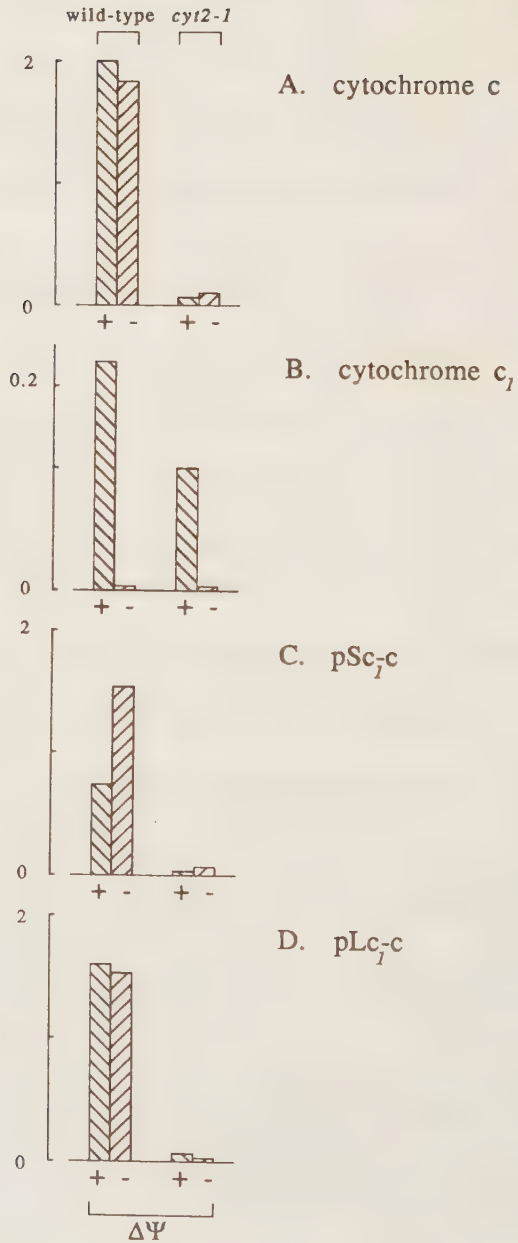
The [ $^{35}\text{S}$ ]cysteine-labelled cytochrome  $c_1$  and Lc $_J$ -c precursor were imported separately into mitochondria to their intermediate-size form in two pools equivalent to 50  $\mu\text{g}$  mitochondrial protein per sample in the presence of 2.5 mM  $\text{MgCl}_2$  for 20 min at 25  $^\circ\text{C}$  as described in Methods. After treatment with 20  $\mu\text{g}$  proteinase K/ml, mitochondria were reisolated by centrifugation and suspended in SEM buffer containing 2 mM PMSF at a protein concentration of 2 mg/ml and portioned into aliquots containing 50  $\mu\text{g}$  mitochondrial protein each. The intermediate-size cytochrome  $c_1$  and Lc $_J$ -c were the predominant species (i.e. > 95 %) accumulated under these import conditions (not shown, see Figures 13A track 2 and Figure 19 lane 2, for comparison). The imported intermediate-size cytochrome  $c_1$  and Lc $_J$ -c were then chased for varying periods of time (as indicated) at 25  $^\circ\text{C}$  in mixtures (100  $\mu\text{l}$  final volume in SEM buffer) in the presence of 1 mg sodium dithionite/ml. After the incubation period, cytochrome  $c_1$  and Lc $_J$ -c were immunoprecipitated and the amounts of holocytochrome  $c_1$  and holo-iLc $_J$ -c formed were determined as described in Methods.

(CCHL), the enzyme which catalyzes heme addition to apocytochrome *c* (Dumont *et al.*, 1987; Nargang *et al.*, 1988). It was then investigated which heme lyase, CC<sub>1</sub>HL or CCHL, was responsible for the covalent attachment of heme to both the pSc<sub>1</sub>-*c* and pLc<sub>1</sub>-*c* fusion proteins. In order to do this, isolated *cyt2-1* mitochondria were employed, these mitochondria are deficient in CCHL activity (Nargang *et al.*, 1988), thus any holo-formation occurring in these mitochondria must be as a result of CC<sub>1</sub>HL activity.

The precursors of cytochrome *c*, cytochrome *c*<sub>1</sub>, and of the fusion proteins, pSc<sub>1</sub>-*c* and pLc<sub>1</sub>-*c* were synthesized in reticulocyte lysate in the presence of [<sup>35</sup>S]cysteine. Mitochondria were isolated from both the wild-type and *cyt2-1* strains of *Neurospora crassa*. Reticulocyte lysate and isolated mitochondria were combined, either in the presence or absence of a membrane potential under reducing conditions, i.e. in the presence of sodium dithionite. Following the incubation all the samples were lysed with 1 % (w/v) Triton X-100 containing buffer and immunoprecipitated, as appropriate, for cytochrome *c*, cytochrome *c*<sub>1</sub>, Sc<sub>1</sub>-*c* and Lc<sub>1</sub>-*c*. HPLC analysis of the resulting tryptic peptides allowed quantification of holo-formation (Figure 27).

Holocytochrome *c* formation occurred in the wild-type mitochondria, independent of a mitochondrial membrane potential, however, no significant CCHL activity was detectable in the *cyt2-1* mitochondria (Figure 27, panel A). The results are thus, in agreement with previous findings (Zimmermann *et al.*, 1979; Nargang *et al.*, 1988). Formation of holocytochrome *c*<sub>1</sub> on the other hand, was dependent on the presence of a  $\Delta\Psi$  (as previously demonstrated) and occurred also in the *cyt2-1* mitochondria, thus demonstrating the presence of CC<sub>1</sub>HL activity in these mitochondria (Figure 27, panel B). Holo-Sc<sub>1</sub>-*c* formation was observed in the wild-type mitochondria and as previously demonstrated was favoured by the absence of a  $\Delta\Psi$ . No significant holo-Sc<sub>1</sub>-*c* formation was detected in the *cyt2-1* mitochondria, which is in agreement with the results presented in Figure 17. The presence or absence of a  $\Delta\Psi$  in wild-type mitochondria, had relatively little effect on the holo-Lc<sub>1</sub>-*c* formation. More importantly, however, no holo-Lc<sub>1</sub>-*c* formation was observed with the *cyt2-1* mitochondria, irrespective of whether the membrane potential was present or not.

Formation of holo  $c$  /  $c_i$  species (fmol.min.mg)<sup>-1</sup>



**Figure 27. pSc<sub>J</sub>-c and pLc<sub>J</sub>-c are not substrates for cytochrome c<sub>1</sub> heme lyase**

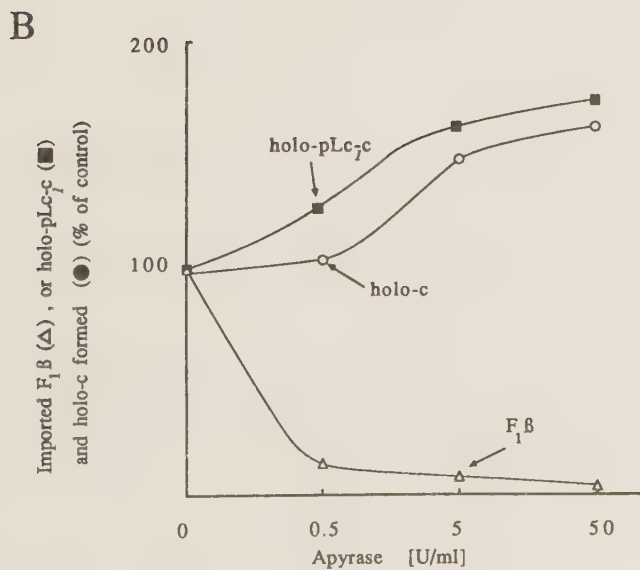
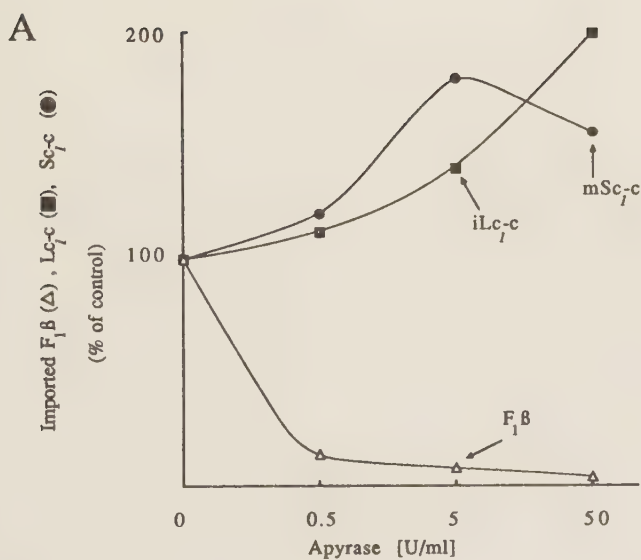
Apocytochrome c, precytochrome c<sub>1</sub>, pSc<sub>J</sub>-c and pLc<sub>J</sub>-c were synthesized in reticulocyte lysates in the presence of [<sup>35</sup>S]cysteine. Mitochondria were freshly isolated from both wild-type and *cyt2-1* *N. crassa* in SEM-buffer containing 2 mM PMSF. The various lysates (20 µl of each) were imported into either wild-type or *cyt2-1* (50 µg protein) in the presence of 2.5 mM MgCl<sub>2</sub>, sodium dithionite (1 mg/ml) and either the presence or absence of antimycin A, oligomycin and valinomycin (as indicated by + or -ΔΨ). Samples were incubated for 20 min at 25 °C and then were lysed with 1 ml of 1 % (w/v) Triton X-100 containing buffer. Levels of holocytochrome c (A), holocytochrome c<sub>1</sub> (B), holo-pSc<sub>J</sub>-c (C) and holo-Lc<sub>J</sub>-c (D) were determined following immunoprecipitation, digestion of immunocomplexes with trypsin and subjection to reverse-phase HPLC, as described in Methods.

In summary, *cyt2-1* mitochondria contained little or no CCHL enzyme activity. It can be concluded that both the pSc<sub>J</sub>-*c* and pLc<sub>J</sub>-*c* fusion proteins are substrates only for CCHL, as no holo-formation of these species was observed in the *cyt2-1* mitochondria. Furthermore these results imply that when imported along the cytochrome *c*<sub>1</sub> route (+ΔΨ), holo-Lc<sub>J</sub>-*c* formation can only occur after an interaction with CCHL has taken place. As it has been demonstrated in Figure 25, that such an interaction cannot occur subsequent to import of pLc<sub>J</sub>-*c* and its processing to iLc<sub>J</sub>-*c* in the matrix, it thus must occur prior to it. As pLc<sub>J</sub>-*c* import into the matrix occurs via contact sites, this strongly suggests that a functional cross-section of the cytochrome *c* heme lyase and the cytochrome *c*<sub>1</sub> import pathway occurs at the contact site area of the mitochondrial membrane system. It is essential therefore to localize the holo-iLc<sub>J</sub>-*c* species.

#### 4.28 Requirement of NTPs for the import of pSc<sub>J</sub>-*c* and pLc<sub>J</sub>-*c* into mitochondria

Nucleoside triphosphates (NTPs) are required for import of precursor proteins into mitochondria and non-hydrolyzable ATP analogues are not able to fulfill this NTP requirement (Pfanner & Neupert, 1986; Pfanner *et al.*, 1987b). It is thus postulated that the hydrolysis of NTPs is involved in modulating the folding of precursors in the cytosol and thereby conferring a conformation on the precursor protein that is more competent for import (see Introduction for a more detailed discussion). All mitochondrial precursor proteins studied to date require an unfolded conformation in order to be translocation competent and hence display a dependence on NTPs for import into mitochondria. In contrast, however, NTPs are not required throughout the import process of cytochrome *c* (Nicholson & Neupert, in preparation), thus a specific folded rather than unfolded conformation of apocytochrome *c* is thought to be required for binding and membrane insertion. It was thus investigated whether import of both pSc<sub>J</sub>-*c* and pLc<sub>J</sub>-*c* into the mitochondrial matrix would be dependent on NTPs or if they shared the characteristic of NTP-independence as shown by cytochrome *c* (Figure 28).

The precursor proteins pSc<sub>J</sub>-*c*, pLc<sub>J</sub>-*c*, apocytochrome *c* and F<sub>1</sub>F<sub>0</sub>-ATPase subunit (F<sub>1</sub>β) were synthesized in reticulocyte lysates in the presence of [<sup>35</sup>S]cysteine except for F<sub>1</sub>β



**Figure 28. Depletion of ATP levels stimulates import of apocytochrome *c* and of pSc<sub>I</sub>-*c* and pLc<sub>I</sub>-*c* into mitochondria**

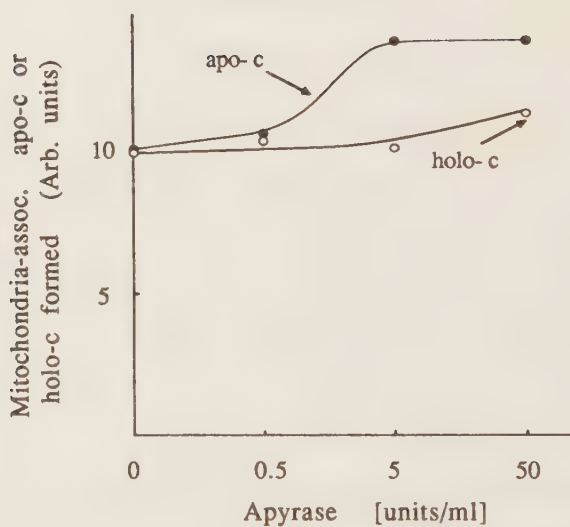
The precursor proteins apocytochrome *c*, pLc<sub>I</sub>-*c* and pSc<sub>I</sub>-*c* and F<sub>1</sub>F<sub>0</sub>-ATPase subunit (F<sub>1</sub>β) were synthesized in reticulocyte lysate in the presence of [<sup>35</sup>S]cysteine except for F<sub>1</sub>β synthesis where [<sup>35</sup>S]methionine was used. Aliquots of apocytochrome *c*, pLc<sub>I</sub>-*c* and pSc<sub>I</sub>-*c* were pooled individually with an aliquot of F<sub>1</sub>β lysate. Lysates were treated with either water, or apyrase at the final concentrations indicated for 15 min at 30 °C followed by 15 min at 25 °C. *Import of pF<sub>1</sub>β*, was tested from an aliquot of each of the treated lysates as follows: lysate (20 μl) was incubated with isolated mitochondria (50 μg protein) in the presence of 2.5 mM MgCl<sub>2</sub>, oligomycin, carboxyatractyloside (20 μM and 2 μM, respectively) and 0.2 mM N,N,N',N'-tetramethylphenylenediamine plus 8 mM K-ascorbate (from a stock adjusted to pH 7.2 with KOH) for 25 min at 25 °C. Samples were cooled to 0 °C and were treated with 20 μg proteinase K/ml for 30 min at 0 °C. Protease activity was halted by the addition of 1 ml of SEM-buffer containing 2 mM PMSF and mitochondria were then reisolated by centrifugation and lysed in a SDS-containing buffer and analysed by SDS/PAGE. *Import of pSc<sub>I</sub>-c and pLc<sub>I</sub>-c into the mitochondrial matrix* (from the remaining part of the respective apyrase-treated lysates) was performed in an identical manner as with pF<sub>1</sub>β, however, following the proteinase K treatment, samples were lysed with Triton X-100 buffer containing 2 mM PMSF. Samples were immunoprecipitated with cytochrome *c*-specific antiserum and immunoprecipitates were dissociated with an SDS-containing buffer and analysed by SDS/PAGE. The radioactive bands corresponding to the mature-size F<sub>1</sub>β, mSc<sub>I</sub>-*c* and iLc<sub>I</sub>-*c* on the resulting gels were visualized by fluorography and quantified by laser densitometry of the resulting films. *Import of apocytochrome c and pLc<sub>I</sub>-c* was performed as follows, the remaining part of the apyrase-treated F<sub>1</sub>β:apocytochrome *c* lysate mixes, or respective F<sub>1</sub>β:pLc<sub>I</sub>-*c* lysate mix, were incubated (20 μl lysate) with isolated mitochondria (50 μg protein in a final volume of 200 μl) in the presence of 3 μM hemin and sodium dithionite (1 mg/ml) for 10 min at 25 °C. Samples were lysed with buffer containing 1 % Triton X-100 and immunoprecipitated with cytochrome specific antiserum. Levels of holocytochrome *c* formed were quantified following HPLC analysis, as described in Methods. The amount of protease-protected mF<sub>1</sub>β, mSc<sub>I</sub>-*c*, iLc<sub>I</sub>-*c*, holo-cytochrome *c* and holo-pLc<sub>I</sub>-*c* formed are expressed as a percentage of the control to which no apyrase was added to the respective lysates.

synthesis where [ $^{35}\text{S}$ ]methionine was used. Aliquots of pSc<sub>I</sub>-c, pLc<sub>I</sub>-c and apocytochrome *c* were pooled separately with an aliquot of F<sub>1</sub>β lysate and all reticulocyte lysate mixtures were treated with increasing concentrations of apyrase. Apyrase is an adenosine 5'-triphosphatase and an adenosine 5'-diphosphatase and hence causes a rapid depletion of exogenous ATP and ADP levels. The treated lysates and isolated mitochondria (preincubated with oligomycin and carboxyatractoside) were then combined under various import conditions. Oligomycin was added to prevent the formation of ATP by the F<sub>1</sub>F<sub>0</sub>-ATPase. Following incubation, import of pSc<sub>I</sub>-c, pLc<sub>I</sub>-c and F<sub>1</sub>β along a ΔΨ-dependent pathway into the mitochondrial matrix was judged by the accumulation of their MPP processed forms in a protease-protected location. Import along the cytochrome *c* pathway of pLc<sub>I</sub>-c and apocytochrome *c* was monitored by assaying levels of their respective holo-forms as described before (3.19).

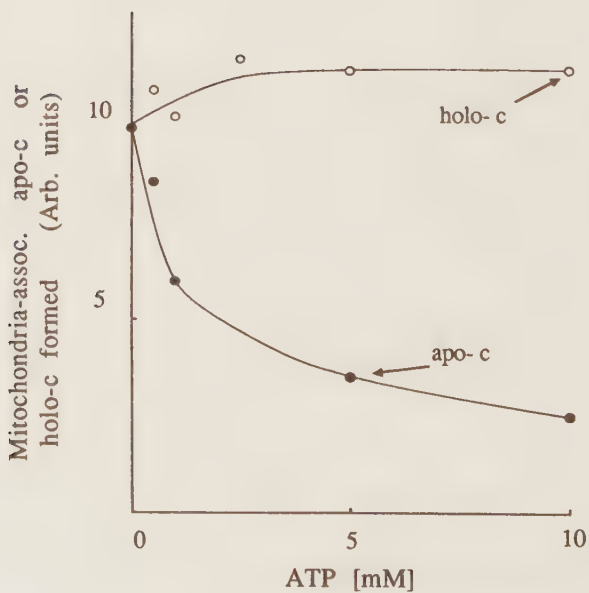
Processing of pF<sub>1</sub>β and import into a protease-protected location was strongly inhibited by apyrase treatment (Figure 28, A and B). As previously demonstrated, holocytochrome *c* formation was stimulated when reticulocyte lysate containing [ $^{35}\text{S}$ ]-labelled apocytochrome *c* had been treated with apyrase (Nicholson & Neupert, in preparation) (Figure 28 A). Unlike F<sub>1</sub>β import, import of pSc<sub>I</sub>-c and pLc<sub>I</sub>-c into the matrix, where they are processed to mSc<sub>I</sub>-c and iLc<sub>I</sub>-c, respectively, however were not inhibited by apyrase pretreatment of the lysate. In fact a stimulation of import was observed at the higher concentrations of apyrase (Figure 28 B). A similar result was obtained for the formation of holo-pLc<sub>I</sub>-c depletion of NTP levels stimulated the import of the fusion protein along the cytochrome *c* pathway (Figure 28 A).

Dissection of the cytochrome *c* import pathway revealed that the initial insertion of apocytochrome *c* into the outer membrane and its binding to cytochrome *c* heme lyase (CCHL) was sensitive to the levels of NTPs. Once bound to mitochondria, chase of apocytochrome *c* to holocytochrome *c* was relatively unaffected by NTP levels (Figure 29 A and B) (Nicholson and Neupert, in preparation). Binding of pSc<sub>I</sub>-c to mitochondria rather than its subsequent chase into the matrix was stimulated by the depletion of NTPs from the import system. A similar result was obtained for the pLc<sub>I</sub>-c fusion protein

A.



B



**Figure 29. In the absence of ATP, binding of apocytochrome *c* to mitochondria, rather than conversion to holocytochrome *c*, is stimulated**

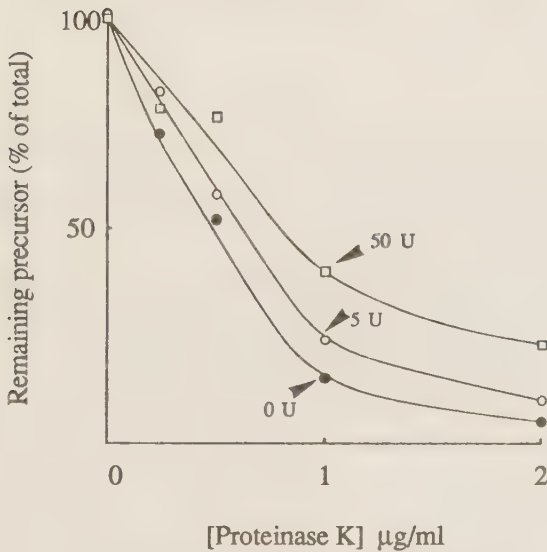
*Binding of apocytochrome *c* to mitochondria.* Reticulocyte lysates containing [<sup>35</sup>S]cysteine-labelled apocytochrome *c* and mitochondria (2.5 mg/ml protein in SEM buffer) were treated (separately) with either water, or apyrase at the concentrations indicated. Reticulocyte lysate treatment was performed by incubating 15 min at 30 °C followed by 15 min at 25 °C, however, the mitochondria treatment was performed for 15 min at 25 °C. The treated lysates and mitochondria were cooled to 0 °C. Binding of the control and apyrase-treated [<sup>35</sup>S]-labelled apocytochrome *c* to the control or apyrase-treated mitochondria was analysed as follows the lysates and the corresponding apyrase-treated mitochondria were combined and incubated in a final volume of 100 µl SEM buffer and incubated at 0 °C for 1 min exactly. The binding reaction was stopped by the dilution of the reaction with 1 ml SEM buffer containing 0.1 M KCl and mitochondria were reisolated by centrifugation. The presence of 0.1 M salt prevents further binding of apocytochrome *c* to mitochondria. Mitochondria were lysed with SDS-containing buffer and analysed by SDS/PAGE and fluorography.

*Conversion of mitochondrial-bound apocytochrome *c* to holocytochrome *c*:* [<sup>35</sup>S]Cysteine-labelled apocytochrome *c* (60 µl reticulocyte lysate) was bound to isolated mitochondria (125 µg protein) in the presence of oligomycin and carboxyatractyloside (20 µM and 2 µM, respectively) in a final volume of 600 µl SEM buffer, for 5 min at 25 °C. The sample was diluted two-fold with SEM buffer and mitochondria were reisolated by centrifugation for 12 min at 17,400 × *g*. Mitochondria with bound [<sup>35</sup>S]-labelled apocytochrome *c* were resuspended in 60 µl SEM buffer, were divided into 10 µl aliquots and were treated with apyrase for 5 min at 25 °C at the concentrations indicated. Samples were cooled to 0 °C and bound apocytochrome *c* was chased to holocytochrome *c* by the addition of 3 µM hemin and sodium dithionite (1 mg/ml) in the presence of oligomycin and carboxyatractyloside (20 µM and 2 µM, respectively) at 0 °C for 1 min exactly. The reaction was stopped precisely by the addition of 1 ml 1% (w/v) Triton X-100 buffer containing the oxidant K<sub>3</sub>[Fe(CN)<sub>6</sub>].

(results not shown). The import pathways of pSc<sub>I</sub>-c and pLc<sub>I</sub>-c into the matrix thus display the same NTP-independence as their import into the intermembrane space along the cytochrome *c* route.

Thus the precursor proteins, pSc<sub>I</sub>-c and pLc<sub>I</sub>-c, would appear to be more translocation competent (for both the cytochrome *c*- and matrix-import pathways) when NTP levels are depleted. All proteins which are translocated across biological membranes are considered to have a more translocation-competent conformation when they have an unfolded structure rather than when they have adopted the "foldedness" of their mature counterparts. This translocation-competent conformation is correlated with a certain looseness or openness of structure which is characterized by an increased sensitivity to proteolytic digestion (Randall & Hardy, 1986; Pfanner et al., 1987b). In contrast, however, upon depletion of NTP levels, the more translocation-competent structure which is adopted by apocytochrome *c*, is a more folded structure, as judged by protease-resistance (Nicholson & Neupert, in preparation). The increase in translocation competence of pSc<sub>I</sub>-c following depletion of NTPs due to apyrase treatment of lysates, like for apocytochrome *c* was correlated with the adoption of a more folded structure as indicated by an increase in resistance to proteinase K digestion (Figure 30).

In summary, in the absence of NTPs, apocytochrome *c* adopts a more folded conformation which is resistant to proteolytic digestion. Apocytochrome *c*, unlike all other mitochondrial precursor proteins studied to date, requires a more folded rather than unfolded conformation to be translocation-competent. This specific conformation is essential for membrane insertion and binding to CCHL, folding following heme addition and translocation across the outer mitochondrial membrane is not affected by depletion of NTP levels. Both pSc<sub>I</sub>-c and pLc<sub>I</sub>-c are also more translocation-competent when they have adopted a more folded structure as a result of depletion of NTP levels following apyrase treatment. Thus it may be concluded that the presence of the apocytochrome *c* domain in the fusion protein not only enables them to insert initially into the outer membrane in a receptor/GIP independent manner, but also by doing so relieves them of the NTP-dependence displayed by all other proteins which are imported into mitochondria. This observation would support the hypothesis that the NTP-mediated



**Figure 30.** Depletion of ATP (and ADP) from reticulocyte lysate containing *in vitro* synthesized pSc<sub>J</sub>-c, decreases pSc<sub>J</sub>-c sensitivity to proteinase K.

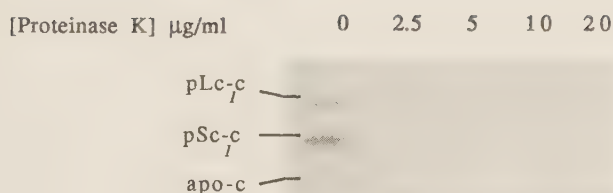
Reticulocyte lysate containing [<sup>35</sup>S]methionine-labelled pSc<sub>J</sub>-c was treated either with water (●), or apyrase at 5 (○) or 50 (□) units/ml by incubation for 15 min at 30 °C followed by 15 min at 25 °C. The treated lysates were cooled to 0 °C and divided into aliquots (5 μl each). Proteinase K was then added at the indicated final concentrations and samples were incubated for 30 min at 0 °C. SDS-containing sample buffer was then added to each sample and all samples were resolved on polyacrylamide gels. The radioactive bands of the resulting gels were visualized by flurography and were quantified by laser densitometry of the resulting film. The amount of undigested precursor protein is expressed as a percentage of the control to which no proteinase K was added. The concentrations of apyrase for pretreatment of the reticulocyte lysate are indicated by arrows (units/ml).

effects are correlated with the maintenance of precursors in an unfolded conformation for initial membrane insertion, rather than receptor binding or later stages of membrane translocation.

#### 4.29 Protease sensitivity of the heme containing *Lc<sub>I</sub>-c* and *Sc<sub>I</sub>-c* fusion proteins

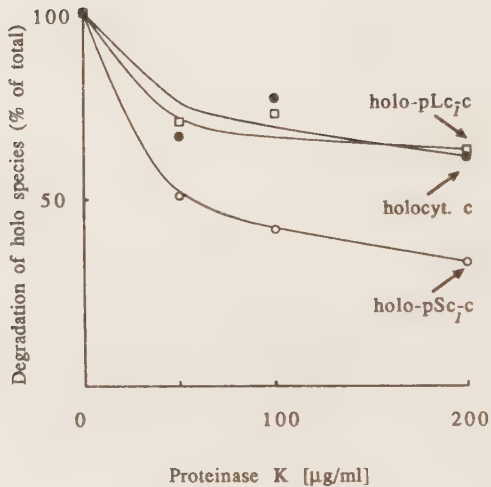
The attachment of heme to apocytochrome *c* has been demonstrated to be a prerequisite for complete translocation of the cytochrome *c* polypeptide across the outer membrane to the intermembrane space. Acquisition of the heme group then is coupled to a folding of the molecule into its native functional conformation (Fisher *et al.*, 1973). Unlike apocytochrome *c*, the correctly folded holocytochrome *c* is very protease resistant, even in the presence of 1 % (w/v) Triton X-100 detergent, the majority cytochrome *c* could not be digested by 200 µg proteinase K/ml, whereas approximately 40-fold less levels are required to degrade the heme-free species, apocytochrome *c* (Nicholson *et al.*, 1988). As previously demonstrated the p*Sc<sub>I</sub>-c* and p*Lc<sub>I</sub>-c* fusion proteins could be accumulated in mitochondria as heme containing species namely, holo-p*Sc<sub>I</sub>-c*, holo-p*Lc<sub>I</sub>-c* and holo-i*Lc<sub>I</sub>-c*. It was therefore interesting to investigate whether the cytochrome *c* domain of these holo-p*Sc<sub>I</sub>-c* and holo*Lc<sub>I</sub>-c* species was correctly folded into a protease-resistant domain, despite the presence of their respective foreign presequences.

Apocytochrome *c*, p*Sc<sub>I</sub>-c* and p*Lc<sub>I</sub>-c* were synthesized in the presence of [<sup>35</sup>S]cysteine in reticulocyte lysate and the proteinase K sensitivity of these precursors in the presence of 1 % (w/v) Triton X-100 was examined (Figure 31). All three precursors were very sensitive to proteinase K, 5 µg proteinase K was sufficient to observe complete degradation of the precursor proteins. [<sup>35</sup>S]-labelled p*Lc<sub>I</sub>-c*, p*Sc<sub>I</sub>-c* and apocytochrome *c* were imported (separately) into isolated mitochondria in the absence of a membrane potential but in the presence of sodium dithionite (i.e. imported along a cytochrome *c* pathway). Following proteinase K treatment, in order to digest non-imported precursor, mitochondria were lysed with a 1 % (w/v) Triton X-100 containing buffer. Each reaction mixture was divided and treated with increasing concentrations of proteinase K as indicated in Figure 32. Following incubation, protease activity was halted by the addition



**Figure 31. Proteinase K sensitivity of the apo-precursor forms of pSc<sub>I</sub>-c, pLc<sub>I</sub>-c and of cytochrome c**

Apocytochrome *c*, pSc<sub>I</sub>-c and pLc<sub>I</sub>-c were synthesized in reticulocyte lysate in the presence of [<sup>35</sup>S]cysteine. The lysates were pooled and treated with Triton X-100 containing buffer (1 % (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris-HCl, pH 7.2) and were divided into aliquots. Proteinase K was then added at the indicated final concentrations and samples were incubated for 30 min at 0 °C. Protease-activity was halted by the addition of PMSF (2 mM) and the undigested precursors were immunoprecipitated together by the addition of apocytochrome *c*-specific antisera. Samples were analysed by SDS/PAGE and fluorography. The bands corresponding to pLc<sub>I</sub>-c, pSc<sub>I</sub>-c and apocytochrome *c* in the resulting film are indicated.

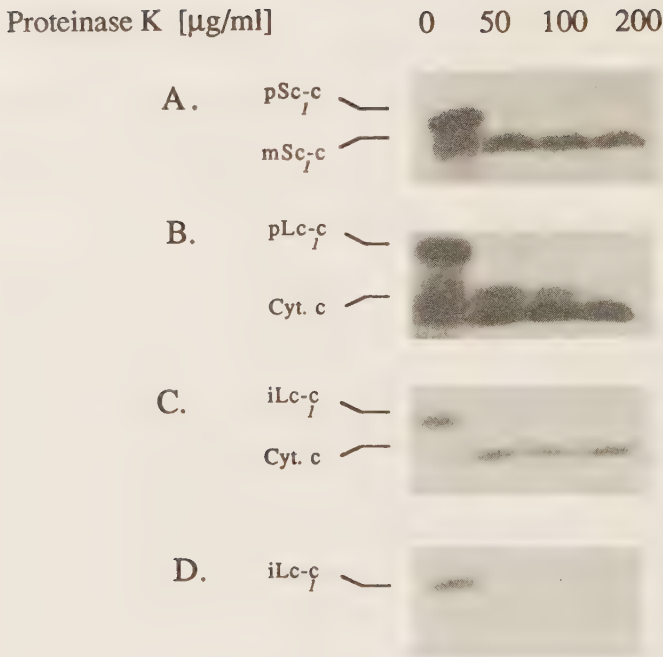


**Figure 32. Proteinase K sensitivity of the holo-forms of cytochrome *c* and of the pSc<sub>I</sub>-*c* and pLc<sub>I</sub>-*c* fusion proteins.**

Apocytochrome *c*, pSc<sub>I</sub>-*c* and pLc<sub>I</sub>-*c* were synthesized in reticulocyte lysate in the presence of [<sup>35</sup>S]cysteine. The [<sup>35</sup>S]cysteine-labelled precursors (50 μl of each lysate) were imported (separately) into isolated mitochondria (250 μg protein, 500 μl final volume) in the presence of 3 μM hemin, antimycin A, oligomycin and valinomycin and sodium dithionite (1 mg/ml) as described in Methods, in order to generate the respective holo-forms. Samples were incubated for 10 min at 25 °C, cooled to 0 °C and treated with 20 μg proteinase K/ml, in order to digest non imported precursor. Protease-activity was halted by the addition of 1 ml SEM buffer containing 2 mM PMSF. Mitochondria were reisolated and lysed in 500 μl of 1 % Triton X-100 buffer, remaining membranes were pelleted by centrifugation at 17,400 × *g* for 15 min. Aliquots of the supernatant (100 μl) were treated with proteinase K at the indicated final concentrations for 30 min at 0 °C. Protease activity was then halted by the addition of PMSF (2 mM) and holo-pLc<sub>I</sub>-*c*, holo-pSc<sub>I</sub>-*c* and holocytochrome *c* species were immunoprecipitated. Levels of holocytochrome *c* (●), holo-pLc<sub>I</sub>-*c* (□) and holo-pSc<sub>I</sub>-*c* (○) were quantified by HPLC analysis as described in Methods.

of PMSF and levels of holo-pLc<sub>1</sub>-c, holo-pSc<sub>1</sub>-c and holocytochrome *c* were quantified by HPLC analysis. The obtained results demonstrated that holo-pLc<sub>1</sub>-c and holo-pSc<sub>1</sub>-c exhibited a similar resistance to protease digestion as that of holocytochrome *c*. In all cases levels of proteinase K required to digest their apo-precursor counterparts had little or no effect on the degradation of the holo signal (Figure 32). These results thus suggest that the cytochrome *c* domain of the fusion proteins can fold into a protease-resistant form upon the covalent attachment of the heme group.

Upon folding of the cytochrome *c* domain in holo-Lc<sub>1</sub>-c and holo-pSc<sub>1</sub>-c, one could imagine two possibilities for the fate of the foreign presequence present in these fusion proteins, namely, either they remain as separate domains outside of the folded holo domain, or somehow they could become "integrated or folded" into the cytochrome *c* domain during its folding process. pLc<sub>1</sub>-c was imported into mitochondria either along a cytochrome *c* pathway (in the absence of a membrane potential, but presence of reductants) or along a cytochrome *c*<sub>1</sub> pathway in the absence or presence of reductants. Import of pSc<sub>1</sub>-c along a cytochrome *c* pathway into mitochondria was also performed. Following proteinase K treatment, mitochondria were reisolated and lysed with 1 % (w/v) Triton X-100 containing buffer. The proteinase K sensitivity of the accumulated Lc<sub>1</sub>-c and Sc<sub>1</sub>-c species was tested once more, but this time proteolytic degradation was monitored by SDS-PAGE rather than by HPLC. When pSc<sub>1</sub>-c was imported in the absence of a membrane potential but in the presence of sodium dithionite, holo-pSc<sub>1</sub>-c accumulated. Upon protease treatment of the detergent solubilized holo-pSc<sub>1</sub>-c a molecular weight shift of pSc<sub>1</sub>-c to its mature-size mSc<sub>1</sub>-c (namely, cytochrome *c*) was observed (Figure 33 A). This species was resistant upto 200 µg proteinase K/ml, thus suggesting that the cytochrome *c* domain of the Sc<sub>1</sub>-c fusion protein was folded and consequently protease resistant. A similar result was obtained when either Triton X-100 solubilized holo-pLc<sub>1</sub>-c or holo-iLc<sub>1</sub>-c were tested for their protease-sensitivity (Figure 33, B and C). However, the accumulated apo-iLc<sub>1</sub>-c was completely protease-sensitive under these conditions (Figure 33D).



**Figure 33.** Upon heme addition, the cytochrome *c* domain of pLc<sub>I</sub>-c, iLc<sub>I</sub>-c and pSc<sub>I</sub>-c folds independently of the presequence

[<sup>35</sup>S]Cysteine-labelled pSc<sub>I</sub>-c and pLc<sub>I</sub>-c were accumulated in mitochondria as their corresponding holo-forms in a protease-protected location, as described above for Figure 32. Holo-iLc<sub>I</sub>-c and apo-iLc<sub>I</sub>-c were also accumulated in mitochondria by importing pLc<sub>I</sub>-c in the presence of a membrane potential and either in the presence or absence of sodium dithionite, respectively. Reisolated mitochondria were lysed with 1 % Triton X-100 containing buffer, the detergent extract was divided into aliquots and treated with proteinase K at the end concentrations indicated for 30 min at 0 °C. PMSF (2 mM) was added to prevent further proteolytic activity and samples were immunoprecipitated with cytochrome *c*-specific antisera. All samples were analysed by SDS/PAGE and fluorography of the resulting gel is presented. The bands corresponding to holo-pSc<sub>I</sub>-c (A), holo-pLc<sub>I</sub>-c (B), holo-iLc<sub>I</sub>-c (C) and apo-iLc<sub>I</sub>-c (D), and the protease-resistant fragment, termed cytochrome *c* because it had the same apparent molecular weight as cytochrome *c* (results not presented) are indicated.

It may be concluded from these results that the presence of foreign sequences at the amino terminus of cytochrome *c* does not hinder the folding of the cytochrome *c* domain upon the covalent attachment of the heme moiety. Folding of the cytochrome *c* domain occurs independently of the presequence, which remains external to the folded holocytochrome domain in a protease-accessible location.

#### **4.30 Investigation of the requirement of the carboxy terminus of apocytochrome *c* for membrane-insertion activity**

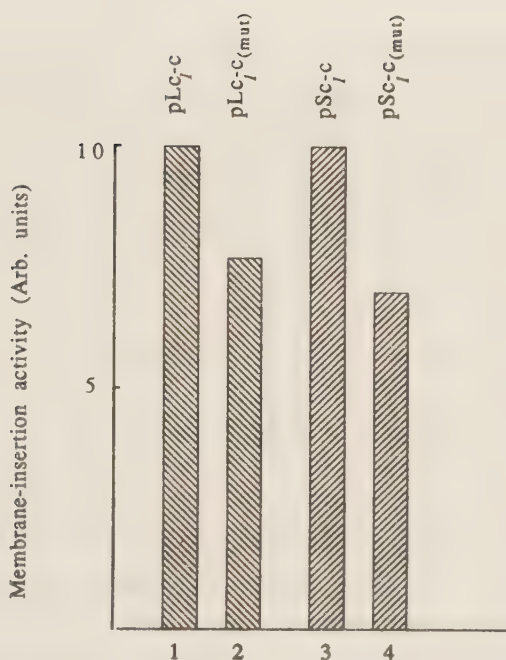
As previously discussed, apocytochrome *c* possesses the ability to spontaneously insert into the outer mitochondrial membrane due to its "membrane-active nature". In the fusion proteins, pSc<sub>1</sub>-*c* and pLc<sub>1</sub>-*c*, the apocytochrome *c* domain exerts its membrane-insertion activity in such a manner that the matrix-targeting sequence gains access to the membrane potential-dependent step and as a result the matrix-targeting domain triggers translocation of the fusion protein across the inner mitochondrial membrane. Therefore due to the presence of the apocytochrome *c* membrane activity, pSc<sub>1</sub>-*c* and pLc<sub>1</sub>-*c* do not require the surface receptor/GIP system for entry into the matrix.

Apocytochrome *c* of the *Neurospora crassa* mutant, *cyc1-1*, cannot bind to mitochondria *in vitro* (Stuart *et al.*, 1987). It was interesting therefore to investigate whether the membrane active nature of the cytochrome *c* domain was adversely affected by this alteration present at the carboxy terminus of the polypeptide. This question was thus addressed by the construction of two new fusion proteins, pSc<sub>1</sub>-*c*(mut) and pLc<sub>1</sub>-*c*(mut). These fusion proteins were constructed in an identical manner as described previously for their wild-type equivalents (4.10) except that the *Hinf*I fragment of the *cyc1-1* cytochrome *c* cDNA instead of the corresponding wild-type *Hinf*I cDNA fragment was ligated into the pGEM4 vector containing the cytochrome *c*<sub>1</sub> cDNA coding for either the matrix-targeting domain of the presequence (pSc<sub>1</sub>-*c*(mut)) or the complete presequence (pLc<sub>1</sub>-*c*(mut)). The ability of these fusion proteins to be imported into protease-treated mitochondria was then tested. Mitochondria isolated from the *cyt2-1* mutant of *Neurospora crassa* were employed so the ability, or lack of it, to interact with CCHL could not play a role in the observed membrane-insertion activity of both the wild-type or

*cyc1-1* cytochrome *c* domains. Hence the ability of the cytochrome *c* domain to mediate the import of the various fusion proteins (wild type and *cyc1-1* mutant) into the matrix (as judged by processing by MPP) in the absence of surface receptors and a functional cytochrome *c* import machinery was used to indicate the "membrane-insertion activity" of the cytochrome *c* polypeptide.

The four fusion proteins were synthesized in reticulocyte lysates in the presence of [<sup>35</sup>S]methionine and were incubated with protease pretreated *cyt2-1* mitochondria in the presence of 2.5 mM MgCl<sub>2</sub> at 25 °C. Import into the matrix of mitochondria was demonstrated by processing of the pSc<sub>1</sub>-*c* and pLc<sub>1</sub>-*c* (wild-type and *cyc1-1* mutant) fusion proteins to their respective mSc<sub>1</sub>-*c* and iLc<sub>1</sub>-*c* forms, respectively. Samples were analysed by SDS/PAGE followed by fluorography, in order to quantify the levels of MPP processed species. Like wild-type apocytochrome *c*, *cyc1-1* apocytochrome *c* was able to mediate the import of the fusion proteins into the matrix of *cyt2-1* mitochondria (Figure 34). Although the import efficiencies of pSc<sub>1</sub>-*c*<sub>(mut)</sub> and pLc<sub>1</sub>-*c*<sub>(mut)</sub> were slightly lower in comparison with their respective wild-type counterparts, it may be concluded that *cyc1-1* apocytochrome *c* still possesses the ability to spontaneously insert into the outer mitochondrial membrane. Furthermore, the import of pSc<sub>1</sub>-*c*<sub>(mut)</sub> and pLc<sub>1</sub>-*c*<sub>(mut)</sub>, like their wild-type equivalents, into the mitochondrial matrix was shown to be independent of GIP (results not shown). The ability of the pSc<sub>1</sub>-*c*<sub>(mut)</sub> and pLc<sub>1</sub>-*c*<sub>(mut)</sub> fusion proteins to be imported along a cytochrome *c* pathway into the intermembrane space was also tested, however, both fusion proteins failed to be imported along this pathway (results not shown).

These results, taken together with the previous results (4.9) would suggest that an intact carboxy-terminus of the apocytochrome *c* polypeptide is essential for the specific binding of apocytochrome *c* to mitochondria, where it forms a complex which includes CCHL, rather than for its initial insertion into the mitochondrial outer membrane



**Figure 34. The carboxy terminus of apocytochrome *c* is not required for membrane-insertion activity**

The four fusion proteins, pSc<sub>I</sub>-c, pLc<sub>I</sub>-c, pSc<sub>I</sub>-c<sub>(mut)</sub> and pLc<sub>I</sub>-c<sub>(mut)</sub> were synthesized in reticulocyte lysates in the presence of [<sup>35</sup>S]methionine. Mitochondria were isolated from *cyt2-1 N. crassa* and were treated with 20 µg proteinase K/ml as described in Methods. Each of the reticulocyte lysates (10 µl) were incubated with mitochondria (50 µg), 2.5 mM MgCl<sub>2</sub> at an end volume of 100 µl BSA buffer, containing 1 mM PMSF. Samples were incubated for 20 min at 25 °C, after which all samples were diluted by the addition of 400 µl SEM-buffer containing PMSF (2 mM). Mitochondria were reisolated by centrifugation and were lysed in SDS-containing buffer and all samples were resolved by SDS/PAGE. The bands corresponding to mSc<sub>I</sub>-c and iLc<sub>I</sub>-c for both wild-type and mutant fusions from a fluorograph of the resulting gel were quantified by laser densitometry. The levels of each precursor employed in the import reaction were also quantified by analysing an aliquot of each of the lysates used on the same SDS/PAGE gel as above. The pSc<sub>I</sub>-c and pLc<sub>I</sub>-c from the resulting fluorographs were also quantified as above. The membrane-insertion activity of each fusion protein was calculated as follows: the levels of MPP-processed (i.e. mSc<sub>I</sub>-c of iLc<sub>I</sub>-c, both mutant and wild-type) species were calculated as a percentage of the total precursor employed in the import reactions. The resulting percentages for both the pLc<sub>I</sub>-c (1) and pSc<sub>I</sub>-c (3) (wild-types) were each set to an arbitrary value of 10 and the percentage of the mutant fusion proteins existing as iLc<sub>I</sub>-c<sub>(mut)</sub> (2) and mSc<sub>I</sub>-c<sub>(mut)</sub> (4) were calculated accordingly.

## 5 DISCUSSION

To ensure the specific and efficient delivery of proteins to various cellular subcompartments, the cell has developed a number of devices to control the targeting of proteins to their correct functional destination. These control systems involve both features of the precursor proteins and components of the destination organelles. The accuracy and specificity of these control systems is vital for the maintenance of the cell, as mistargeting of proteins *in vivo* would be fatal. In the case of mitochondrial protein import, most precursor proteins carry amino-terminal extension sequences which contain mitochondrial targeting information and receptors exist on the mitochondrial surface to recognize and bind precursor proteins destined for that organelle.

Import of cytochrome *c*, however, does not share many features in common with the above mentioned general targeting mechanism displayed by other mitochondrial precursor proteins. Apocytochrome *c* does not contain such a targeting sequence, for example. How then is apocytochrome *c* specifically targeted to mitochondria? To address this question a *Neurospora crassa* mutant, *cycl-1*, deficient in cytochrome *c* was studied. Previous investigations had concluded that the defect in cytochrome *c* import in the *cycl-1* mutant was due to a problem with the cytochrome *c* polypeptide itself, rather than a fault in the mitochondrial import machinery (Hennig & Neupert, unpublished results). To investigate the specific details of this *cycl-1* apocytochrome *c* it was essential to obtain cDNA clones not only for the *cycl-1* cytochrome *c* but also for the wild-type cytochrome *c*, so that they could be compared. The similarities and differences of the wild-type cytochrome *c* mRNA and protein, compared to those of the *cycl-1* cytochrome *c* are discussed with relevance to the targeting of apocytochrome *c* to mitochondria.

Secondly, it was investigated as to what special endogenous property the apocytochrome *c* polypeptide has that enables it to discard all the classical features of mitochondrial protein import (precursor presequences, surface receptors, GIP-dependent translocation) and take a completely unique import pathway into mitochondria. In order to do this a series of fusion proteins between apocytochrome *c* and mitochondrial presequences were constructed. The mitochondrial import pathways of these fusion proteins were studied. The presence of the apocytochrome *c* domain in these fusion proteins bestowed a unique

property on the precursors, namely, the ability to circumvent many classical steps of import into the mitochondrial matrix such as surface receptor binding, membrane insertion by GIP and NTP-mediated precursor unfolding. The importance of these results are discussed with reference not only to the unique features of the apocytochrome *c* precursor protein, but also to how these special attributes of the precursor may have played a significant role in the evolution of cytochrome *c* import.

### 5.1 Deficiency in mRNA splicing in a cytochrome *c* mutant of *Neurospora crassa*

The amino acid sequence of wild-type *Neurospora crassa* cytochrome *c* deduced from the cDNA sequence confirmed the previously published protein sequence and the absence of an AUG codon upstream from the postulated initiation codon confirmed that the apocytochrome *c* precursor is synthesized without a cleavable amino-terminal extension sequence (Zimmermann *et al.*, 1981). Comparison of the *N. crassa* cytochrome *c* mRNA sequence (coding region) with that of iso-1-cytochrome *c* from *S. cerevisiae* (Boss *et al.*, 1981) revealed a high level of homology (~65 %). *N. crassa*, like chicken (Limbach & Wu, 1983) and *Schizosaccharomyces pombe* (Russell & Hall, 1982), contains only one functional cytochrome *c* gene per haploid genome. It has been previously demonstrated that *Drosophila melanogaster* (Limbach & Wu, 1985b) and *S. cerevisiae* (Montgomery *et al.*, 1980) contain two cytochrome *c* genes per haploid genome. In contrast, mammals seem to contain ~20-30 cytochrome *c*-like sequences (Scarpulla *et al.*, 1982), the majority appear to be non-functional pseudogenes (Scarpulla & Wu, 1983).

Sequence analysis of the cDNA from the cytochrome *c*-deficient mutant, *cyc1-1*, revealed the presence of a larger open reading frame, owing to the presence of a 74-bp intervening sequence in the 3' area of the coding region. The transcript codes for a protein with an extra 19 amino acids at the carboxy terminus, when compared to the wild-type cytochrome *c* protein. The presence of a GUACAU sequence at the 5' end of the intervening sequence and a CAG sequence at the 3' junction led to the suspicion that the mutant cytochrome *c* mRNA might contain an unspliced intron. Consistent with this view, a sequence CUAUC was observed, resembling the conserved branch sequence, 14

nucleotides upstream from the 3' junction. To date, it has been demonstrated that only chicken (Limbach & Wu, 1983), mouse (Limbach & Wu, 1985a) and rat (Scarpulla *et al.*, 1981) cytochrome *c* genes contain one intron in their coding regions. These introns would appear to be conserved as they interrupt all three genes at identical positions (between codons 55 and 56 of the coding region) and also appear to be of comparable lengths (Limbach & Wu, 1985a), however, no intron present in the 3' area of the coding region of a cytochrome *c* gene has been observed to date. Sequence analysis confirmed that the *N. crassa* cytochrome *c* gene contains an intron of 74-bp after codon 102. The 5' donor splice sequence, GTACGT, the 3' acceptor sequence, CAG, and the branch sequence, CTAAC, all conform to consensus sequences derived from other *N. crassa* introns (Arends & Sebald, 1984; Gurr *et al.*, 1987; Tropschug *et al.*, 1988). These conserved sequences in the wild-type intron, however, differ from those found in the mutant intervening sequence. The mutant cytochrome *c* mRNA contains the sequence GUACAU instead of GUACGU at the 5' splice site and the sequence CUAUC instead of CUAAC at the branch site. Splicing of the intron from the mutant mRNA is thus prevented or retarded owing to the presence of these two base changes.

The compilation of consensus sequences at both the 5' (donor) and 3' (acceptor) splice sites of introns and detailed analysis of the RNA generated during pre-mRNA splicing *in vivo* and *in vitro* have served to increase our understanding on the mechanism of pre-mRNA splicing. In both yeast (*S. cerevisiae*) and mammalian cells (Ruskin *et al.*, 1984; Padgett *et al.*, 1984), a two step model has been proposed. First, cleavage at the 5'-splice site and the lariat (branch point) formation occurs, where the 5' end of the intervening sequence is attached to the branch point via a 2'-5' phosphodiester linkage. This step is followed by cleavage at the 3' splice junction and ligation of the two exons to produce the spliced mRNA occurs. The intervening sequence is therefore released as a lariat. In yeast, the sequence which serves as the site of lariat formation TACTAAC, is strictly conserved (Domdey *et al.*, 1984; Rodriguez *et al.*, 1984), likewise the 5' donor splice sequence GTAPyGT, is essentially completely conserved (Langford & Gallwitz, 1983; Teem *et al.*, 1984). Of these two conserved sequences, the first guanosine of the 5' splice junction and the last adenosine of the branch sequence are particularly important, as

mutations at these two positions, i.e. the sites of bond cleavage and branch formation, dramatically affect splicing (Newman *et al.*, 1985).

In the branch sequence of the *cyc1-1* cytochrome *c* mRNA intron, the final A residue, observed in the wild-type genomic sequence, is changed to a U residue. We speculate, therefore, that the formation of the 2'-5' phosphodiester bond with the first G residue of the splice sequence is prevented, thus the essential lariat formation does not occur.

Two roles for the 5' consensus sequence, GTAPyGT, have been postulated (Parker & Guthrie, 1985; Newman *et al.*, 1985), the first being to define the 5' splice site and the second to ensure that the correct branch sequence is formed. A mutation in a yeast intron has been reported, where a G to A transition at the fifth position of the consensus sequence GTAPyGT had an adverse effect on intron removal (Parker & Guthrie, 1985). Thus the G residue in position 5 would seem to be of particular importance in the splicing process. In support of this, with the exception of the invariant GT, this G residue is the most highly conserved nucleotide, in mammals (84 %) within the 5' consensus sequence (Mount, 1982). In the mutant cytochrome *c* mRNA it is this G residue in position 5 which is mutated to an A residue, thus it would probably serve to inhibit the 5' endonucleolytic cleavage of the 5' junction if the lariat formation had been allowed to occur. However, some correctly spliced mRNA accumulates in the *cyc1-1* cells, this may indicate unofficial usage of alternative sites in the intron consensus sequences.

## 5.2 The importance of the carboxy terminus for the import of apocytochrome *c* into mitochondria

As a consequence of this defective mRNA splicing, *cyc1-1* synthesizes an apocytochrome *c* with an altered carboxy terminus. The polypeptide sequence is changed from amino acid 102 onwards, resulting in an apocytochrome *c* which is 19 amino acids longer than the corresponding wild-type protein, thus the final 27 amino acids are of an unrelated sequence. The mutant apocytochrome *c* is not significantly imported into mitochondria either *in vivo* or *in vitro*; in fact *in vivo* the mutant apocytochrome *c* accumulates in the cytosol (B. Hennig & W. Neupert, unpublished results). The reduction in levels of

holocytochrome *c* present in *cyc1-1* mitochondria is wholly due to this defect in the apocytochrome *c* molecule itself, which renders it incompetent for binding to the mitochondrial surface, rather than due to a defect in the mitochondrial import machinery. Number and affinity of the apocytochrome *c* binding sites have been found to be unaffected in isolated *cyc1-1* mitochondria (results not shown). It was thus concluded that the interaction of the apoprotein with either the outer mitochondrial membrane and/or cytochrome *c* heme lyase is disturbed. A very small amount of mutant holocytochrome *c*, however, is formed *in vivo* and peptide analysis confirms the sequence derived from the cDNA (B. Hennig & W. Neupert, unpublished results).

The altered polypeptide sequence is rather hydrophilic and contains one negative and three positive charges. It is highly remarkable that this relatively minor alteration at the carboxy terminus of apocytochrome *c* confers inability for import, when one considers the diverse passenger proteins (non-mitochondrial hydrophilic proteins) which can be efficiently imported into mitochondria if a mitochondrial-targeting sequence is linked to the amino terminus. This is independent confirmation for the view that the import pathway of cytochrome *c* is unique with respect to all other mitochondrial proteins studied to date. Matsuura *et al.*, (1981) postulated that an addressing signal is contained in a cytochrome *c* fragment extended from amino acid residue 66 to the carboxy end of the protein, which serves to target the apocytochrome *c* to mitochondria. In this respect it is also interesting to note the carboxy terminus is the most evolutionary conserved part of the cytochrome *c* polypeptide with the exception of the heme binding region. It cannot be concluded from the findings of the present *cyc1-1* study that a "targeting signal" is located at the carboxy terminus of apocytochrome *c*.

### 5.3 Targeting of apocytochrome *c* to mitochondria

The presequences of many mitochondrial precursor proteins have been shown to contain the necessary information for mitochondrial targeting. Proteinaceous receptors on the surface of mitochondria, with which precursor proteins specifically interact, play a role in "decoding" the targeting information contained in the precursor protein. Hence correct targeting of proteins to mitochondria involves both the targeting information in the

precursor and specific recognition or decoding of this information by receptor sites on the mitochondrial surface. If we consider cytochrome *c* import, however, the situation appears to be very different.

Cytochrome *c* is synthesized as a precursor which does not contain an amino-terminal presequence and no protease-sensitive receptors exist on the surface of the outer mitochondrial membrane to mediate initial binding and import of the apocytochrome *c* precursor. How is apocytochrome *c* specifically targeted to mitochondria? It is proposed here that the first step in cytochrome *c* import is its spontaneous insertion into the outer membrane, namely, the formation of protein-lipid interactions. It is unlikely that there is a high degree of specificity in this step for the mitochondrial outer membrane. However, once inserted into the lipid bilayer, in the case of mitochondria only, apocytochrome *c* forms a tight complex which includes cytochrome *c* heme lyase (CCHL). Evidence to date would suggest that CCHL acts *in lieu* of a surface receptor, that it is involved in both binding apocytochrome *c* in addition to its catalytic conversion to holocytochrome *c*. The lack of involvement of other proteinaceous components in mediating apocytochrome *c* binding cannot as of yet be definitely ruled out, but all the evidence to date (as outlined in the Introduction) would strongly speak against this (Nicholson *et al.*, 1988). This question can only be finally answered when the purified CCHL is reconstituted into liposomes and thereby reconstituting the apocytochrome *c* import system.

It is proposed here that unlike other mitochondrial precursor proteins, no targeting sequence *per se* exists to target apocytochrome *c* to mitochondria. However, a specific overall conformation, i.e. a certain "folded state" of apocytochrome *c*, rather than a targeting sequence, is required for mitochondrial targeting (Nicholson *et al.*, 1988). Binding of apocytochrome *c* to mitochondria involves features of both the amino- and carboxy-regions of the apocytochrome *c* polypeptide. Amino-terminal cysteines 14 and 17 must enter the intermembrane space for interaction with CCHL and subsequent heme linkage. The results presented here also stress the importance of the carboxy terminus in the targeting of apocytochrome *c* to mitochondria. Alterations in the carboxy terminus of apocytochrome *c*, such as that observed in *cyc1-1* apocytochrome *c*, do not significantly affect the ability of the precursor to spontaneously insert into the mitochondrial

membrane. The results obtained here, however, would suggest that an intact carboxy terminus of the apocytochrome *c* polypeptide is essential for the functional binding of apocytochrome *c* to the CCHL. This would also favour the hypothesis that CCHL acts as the apocytochrome *c* binding site in mitochondria. The fact that *cyc1-1* apocytochrome *c* cannot bind functionally to detergent solubilized CCHL, may also account for the reason for its inability to bind to mitochondria.

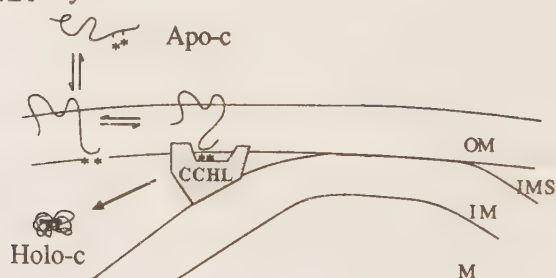
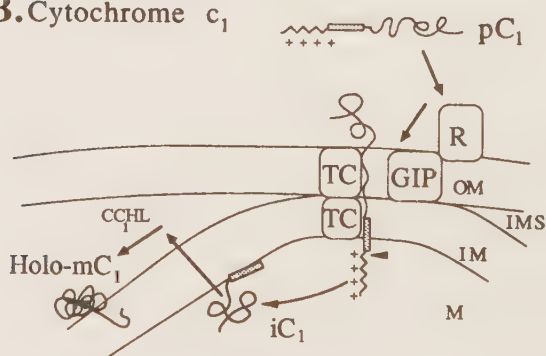
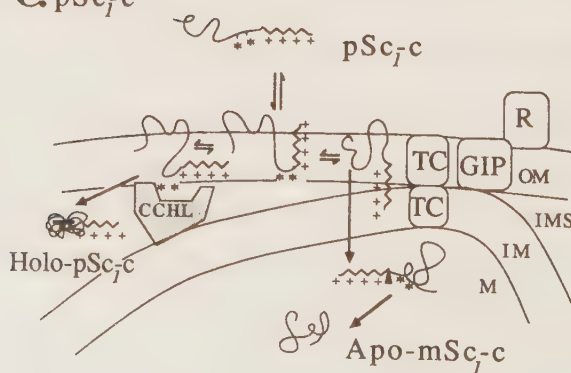
The importance of a specific conformation of apocytochrome *c* for its binding to mitochondria is also supported by the observations that apocytochrome *c* that had been denatured by urea, or cycles of freezing and thawing was not able to insert into membranes (Hennig & Neupert, 1983). These findings complement the recent findings by Nicholson & Neupert, with regard to the role of NTPs in cytochrome *c* import. By depleting NTPs from the import system, one thereby inhibits the NTP-requiring processes that normally function to maintain precursor proteins in an unfolded, translocation-competent form. Hence in the absence of NTPs, apocytochrome *c* folds into a more protease-resistant structure, i.e. it becomes more folded. This "folded" structure was found to be a more import-competent form, in contrast to an unfolded apocytochrome *c* structure. Dissection of the cytochrome *c* pathway, revealed that specific binding to mitochondria was enhanced when the apocytochrome *c* precursor was more "folded", once bound to mitochondria, however, subsequent holocytochrome *c* formation was relatively unaffected by NTP levels.

Together, all results would suggest that a folded, rather than unfolded, conformation of apocytochrome *c* is critical for insertion into the outer mitochondrial membrane. The carboxy terminus of apocytochrome *c* also plays an important role in binding to CCHL. An inability to interact efficiently with CCHL, may thus explain why *cyc1-1* apocytochrome *c* fails to bind with high affinity to mitochondria.

#### 5.4 The mitochondrial import pathways of the pSc<sub>1</sub>-*c* fusion protein

The hybrid protein pSc<sub>1</sub>-*c* is composed of the matrix targeting domain of the cytochrome *c*<sub>1</sub> presequence joined to the amino terminus of apocytochrome *c*. This fusion protein

contains dual targeting information for distinct mitochondrial import pathways and could be selectively imported along either a cytochrome  $c_1$  route into the mitochondrial matrix or via the cytochrome  $c$  into the intermembrane space. The proposed import pathways of pSc<sub>I</sub>- $c$  are depicted in the model in Figure 35 C, where the import pathways of cytochrome  $c$  (Figure 35 A) and cytochrome  $c_1$  (Figure 35 B) are also outlined for comparison. The Sc<sub>I</sub>- $c$  fusion protein is initially synthesized as a precursor, which contains a matrix-targeting sequence. In the presence of a membrane potential this precursor was imported into the mitochondrial matrix where in the presence of Mg<sup>2+</sup> it underwent processing to its mature-size form (mSc<sub>I</sub>- $c$ ), and accumulated as a soluble species in the matrix. The kinetics and efficiencies of this import pathway were similar to those of cytochrome  $c_1$ , thus it may be concluded that the observed import process was not as a result of a low-efficiency non-specific (by-pass) pathway. The targeting information contained in the apocytochrome  $c$  domain was also shown to be "active", namely, it was demonstrated that this protein could be imported along the cytochrome  $c$  pathway and be accumulated in the intermembrane space as an unprocessed pSc<sub>I</sub>- $c$  species, however, that contained a covalently bound heme group. Furthermore, import of pSc<sub>I</sub>- $c$  along the cytochrome  $c$  pathway occurred with comparable efficiency and kinetics as that of the corresponding legitimate parent precursor protein, apocytochrome  $c$ . This pathway occurred in both control and protease-treated mitochondria. The presence of a cytochrome  $c_1$  receptor was not essential for the import of pSc<sub>I</sub>- $c$  into the mitochondrial matrix, as pretreatment of mitochondria with proteases did not significantly hinder the import of pSc<sub>I</sub>- $c$ , in contrast to the import of cytochrome  $c_1$ . Furthermore, the kinetics of import of pSc<sub>I</sub>- $c$  into protease-treated mitochondria were shown to be identical to those into untreated mitochondria. As no quantitative or qualitative difference between the matrix import pathway of pSc<sub>I</sub>- $c$  in either untreated and protease-treated mitochondria could be determined, it could not be conclusively demonstrated whether the pSc<sub>I</sub>- $c$  ever in fact interacted with the cytochrome  $c_1$  receptor throughout its import into the mitochondrial matrix. Initial binding of this fusion protein to mitochondria would therefore, be mediated via the apocytochrome  $c$  domain.

**A. Cytochrome c****B. Cytochrome  $c_1$** **C. pSc<sub>f</sub>-c**

**Figure 35. Hypothetical import and assembly pathways of cytochrome *c*, cytochrome  $c_1$  and pSc<sub>I</sub>-*c***

A. Apocytochrome *c* (Apo-*c*), the precursor of holocytochrome *c* (Holo-*c*), partially inserts into the mitochondrial outer membrane (OM) where it binds to cytochrome *c* heme lyase (CCHL), which acts as specific binding sites *in lieu* of a surface receptor. Translocation across the outer membrane directly into the intermembrane space (IMS) is believed to be driven by the folding of the holocytochrome *c* polypeptide as a result of the covalent heme addition.

B. After synthesis in the cytosol, the precursor of cytochrome  $c_1$  (pC<sub>1</sub>) binds specifically to distinct receptor sites (R) on the mitochondrial surface. Following insertion into the outer membrane (OM), mediated by the general insertion protein (GIP), pC<sub>1</sub> is translocated through contact sites (Translocational contact Sites, TS) into the matrix (M), in a membrane potential-dependent manner. Processing to an intermediate-size cytochrome  $c_1$  (iC<sub>1</sub>) occurs followed by retranslocation across the inner membrane (IM). Conversion of apocytochrome  $c_1$ , thus exposed to the intermembrane space (IMS) to its holo-counterpart, is catalysed by cytochrome  $c_1$  heme lyase (CC<sub>1</sub>HL). The second processing event resulting in the formation of holo mature-size cytochrome  $c_1$  (holo-mC<sub>1</sub>) then ensues.

C. pSc<sub>I</sub>-*c* initially inserts into the mitochondrial outer membrane (OM) due to the membrane active nature of its apocytochrome *c* domain. Bound pSc<sub>I</sub>-*c* exists in an equilibrium from where it can be chased along either a cytochrome *c*- or matrix-import pathway. Upon the addition of a reductant, pSc<sub>I</sub>-*c* could be imported along the cytochrome *c* pathway where it accumulated in the intermembrane space (IMS) as a heme containing species, holo-pSc<sub>I</sub>-*c*. On the other hand, bound pSc<sub>I</sub>-*c* could also be chased into the mitochondrial matrix (M) in a membrane potential-dependent manner, where it was processed to its mature-size form (mSc<sub>I</sub>-*c*) and accumulated as a soluble species in the matrix (Apo-mSc<sub>I</sub>-*c*). The two cysteine residues to which the heme group becomes covalently attached are indicated by asterisks.

The pathway for import into mitochondria that is dependent on apocytochrome *c* insertion properties is not separate or exclusive of the  $\Delta\Psi$ -dependent pathway. Fusion proteins containing signals for both can move between these pathways and are directed one way or the other in a "tug of war" type of reaction. In the case of pSc<sub>J</sub>-*c*, the  $\Delta\Psi$ -dependent pathway to the matrix was dominant over the cytochrome *c* pathway into the intermembrane space when both routes are fully active during ongoing import. However, in protease-treated mitochondria it is postulated that the membrane active domain (namely, the apocytochrome *c* domain) mediates the initial insertion of pSc<sub>J</sub>-*c* into the outer membrane. In the absence of a membrane potential and reductants the pSc<sub>J</sub>-*c* remains bound to mitochondria, accessible to exogenously added proteases whilst forming a salt-resistant complex with cytochrome *c* heme lyase. From this point pSc<sub>J</sub>-*c* can be directed either on a cytochrome *c*- or a matrix-import pathway. The ability of pSc<sub>J</sub>-*c* to initiate import along the cytochrome *c*-pathway but then be redirected to the mitochondrial matrix along the  $\Delta\Psi$ -dependent pathway, demonstrates that some functional intersection of these pathways exist which bridges the exchange of pSc<sub>J</sub>-*c* from one pathway to the other under appropriate conditions.

Attempts to answer the question as to whether import of pSc<sub>J</sub>-*c* into the matrix occurred via contact sites were not successful so far. The approach used was that of prebinding antibodies directed against the apocytochrome *c* domain of the pSc<sub>J</sub>-*c* fusion protein (see also for the pLc<sub>J</sub>-*c* fusion protein, below). A processed translocation intermediate spanning both mitochondrial membranes could not be generated. It was concluded from these results that once the targeting information of the apocytochrome *c* domain was restricted by the bound antibody, the matrix-targeting domain was not of sufficient length to span the contact site region in such a manner that the MPP cleavage site became exposed to the matrix.

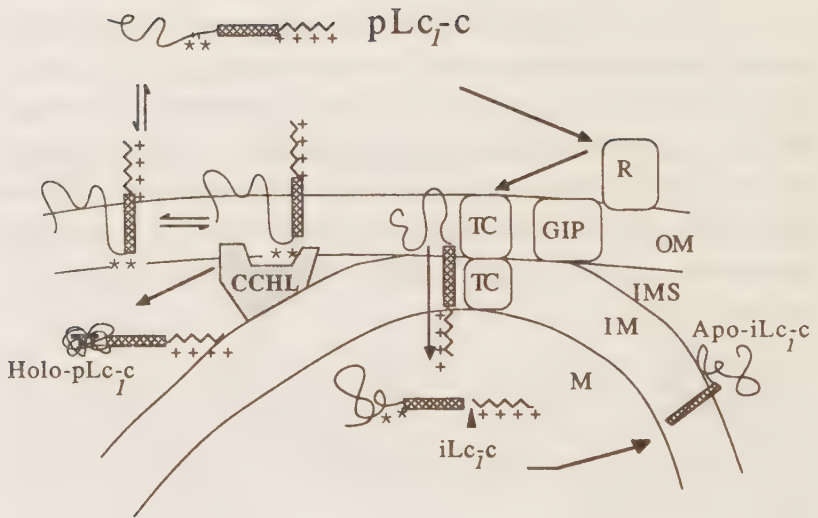
#### 5.4 The import pathways of pLc<sub>J</sub>-*c* into mitochondria

In contrast to pSc<sub>J</sub>-*c*, the pLc<sub>J</sub>-*c* fusion protein is comprised of the complete bipartite structure of the cytochrome *c*<sub>1</sub> presequence joined to the amino terminus of apocytochrome *c*. It has been previously been postulated that the cytochrome *c*<sub>1</sub>

presequence contains not only the necessary information to target this protein to the mitochondria but also to its correct submitochondrial location. The processing of cytochrome  $c_1$  during import into mitochondria appears to occur by the following sequence of events. a) During or following the import of pC<sub>1</sub> into the mitochondrial matrix, the matrix-targeting domain of the bipartite presequence is removed by MPP. b) The iC<sub>1</sub> thus formed is re-exported to the intermembrane-space side of the inner membrane where the heme is covalently linked by the enzyme cytochrome  $c_1$  heme lyase (CC<sub>1</sub>HL). c) The second processing peptidase then removes the remainder of the presequence (the postulated "re-export domain", see below), thereby generating mC<sub>1</sub> that is ready for assembly into the bc<sub>1</sub> complex (van Loon *et al.*, 1986; Hartl *et al.*, 1987; Nicholson *et al.*, 1989).

The proposed import pathways of pLC<sub>1</sub>-c are depicted in Figure 36. The apocytochrome *c* targeting information in pLC<sub>1</sub>-c was also found to be functional and thus pLC<sub>1</sub>-c could also be imported along a cytochrome *c* pathway, which resulted in the accumulation of holo-pLC<sub>1</sub>-c in mitochondria.

A functional dissection of the import pathway of the pLC<sub>1</sub>-c fusion protein (Figure 36) revealed in fact that the cytochrome  $c_1$  presequence does contain all the necessary information to correctly deliver this protein to its final location, namely, at the intermembrane-space side of the inner membrane, when directed along a  $\Delta\Psi$ -dependent import pathway. The amino terminal part of the presequence, the matrix-targeting domain, directed the pLC<sub>1</sub>-c via the contact sites into the mitochondrial matrix in a  $\Delta\Psi$ -dependent manner. If imported at low temperature (8 °C), pLC<sub>1</sub>-c could be accumulated as a soluble matrix protein. In the presence of divalent cations it was correctly processed to its intermediate-size form, iLC<sub>1</sub>-c. Once in the matrix, at higher temperatures (25 °C) the second domain of the cytochrome  $c_1$  presequence directed its carrier protein (apocytochrome *c*) along its ancestral assembly pathway for subsequent intramitochondrial sorting. The apocytochrome *c* domain thus became retranslocated across the inner membrane where, like iC<sub>1</sub> and is accumulated as an apo-intermediate size LC<sub>1</sub>-c species, exposed to the intermembrane space.



**Figure 36. Hypothetical import and assembly pathways of pLc<sub>1</sub>-c**

*A. Import along the cytochrome *c* pathway:* The precursor of Lc<sub>1</sub>-c (pLc<sub>1</sub>-c) initially inserts into the outer mitochondrial membrane (OM) due to the membrane insertion active nature of its apocytochrome *c* domain and forms a salt-resistant complex which includes cytochrome *c* heme lyase (CCHL). Upon the addition of a reductant, pLc<sub>1</sub>-c could be imported along a cytochrome *c* pathway where it accumulated in the intermembrane space (IMS) as a heme-containing species, holo-pLc<sub>1</sub>-c. The two cysteines to which the heme group becomes covalently attached are indicated by asterisks.

*B. Import along the cytochrome *c*<sub>1</sub> pathway:* On the other hand pLc<sub>1</sub>-c could be imported along a cytochrome *c*<sub>1</sub> route, initially into the matrix (M) via translocation contact sites (TC) in a membrane potential-dependent manner. Processing to an intermediate-size Lc<sub>1</sub>-c (iLc<sub>1</sub>-c) occurs, catalysed by the matrix processing peptidase, a reaction dependent on the presence of divalent cations. This process is followed by retranslocation across the inner membrane (IM), whereby iLc<sub>1</sub>-c becomes exposed to the intermembrane space while being anchored to the inner membrane, presumably via the remaining, hydrophobic part of its presequence. Retranslocated iLc<sub>1</sub>-c cannot functionally interact with cytochrome *c*<sub>1</sub> heme lyase.

Based on the endosymbiotic origin of mitochondria, export from the mitochondrial matrix across the inner membrane to the intermembrane space is comparable to the export of proteins from the bacterial cytoplasm across the plasma membrane to the periplasmic space. It had been previously postulated that the second domain of the cytochrome  $c_1$  presequence is responsible for the export of cytochrome  $c_1$  from the matrix to the intermembrane space. This hypothesis was supported by the remarkable similarity that exists between the second part of the mitochondrial cytochrome  $c_1$  presequence and the export sequence of the bacterial cytochromes  $c_1$  and  $c_2$  (Figure 37). Thus it can now be shown directly from these results of the import of the pL $c_1$ - $c$  fusion protein that the hydrophobic domain of the presequence alone does indeed contain the necessary information to act as a "re-export" sequence.

Although the apo-iL $c_1$ - $c$  can be accumulated in the same submitochondrial location as apo-iC $_1$ , from this position, unlike apocytochrome  $c_1$ , it cannot be converted to its holo counterpart and it does not undergo its second processing event to generate a mature-size L $c_1$ - $c$  species (mL $c_1$ - $c$ ). We have recently demonstrated that the second processing event of the apocytochrome  $c_1$  presequence which takes place at the outer face of the inner membrane, occurs only after the covalent heme attachment to cytochrome  $c_1$  has taken place and not before (Nicholson *et al.*, 1989). This reaction is catalysed by cytochrome  $c_1$  heme lyase (CC $_1$ HL). It has been shown here that pL $c_1$ - $c$  is not a substrate for CC $_1$ HL although it is correctly delivered to the environment of CC $_1$ HL. It is concluded, therefore, that as CC $_1$ HL catalysed-holo-formation of the iL $c_1$ - $c$  accumulated in the mitochondria does not take place, the subsequent second processing event also cannot occur, hence formation of mL $c_1$ - $c$  has not been observed. Holo-formation of pL $c_1$ - $c$  is only catalysed by cytochrome  $c$  heme lyase (CCHL). As no holo formation of pL $c_1$ - $c$  occurs following re-export from the matrix to the outer surface of the inner membrane, these results would suggest that CCHL does not appear to be located at the sites of export of proteins from the matrix. In the presence of a membrane potential and reductants (i.e. when both cytochrome  $c$  and  $c_1$  import pathways are active) holo-iL $c_1$ - $c$  accumulated. It is proposed here that holo-L $c_1$ - $c$  formation precedes the processing of pL $c_1$ - $c$  to iL $c_1$ - $c$  by the matrix processing peptidase (MPP).



Figure 37. Sequence comparison of the carboxy-terminal half of the mitochondrial cytochrome *c*<sub>1</sub> presequence with leader peptides of bacterial cytochromes *c*<sub>1</sub> and *c*<sub>2</sub>. The carboxy-terminal half of the mitochondrial cytochrome *c*<sub>1</sub> presequence (amino acids 28-61) is aligned with the leader peptides of *Rhodobacter sphaeroides* cytochrome *c*<sub>1</sub> and *Rhodobacter capsulata* cytochrome *c*<sub>2</sub>. Sequences are taken from Sadler *et al.*, (1984), Gabellini & Sebald, (1986) and Daldal *et al.*, 1986, respectively. Gaps are introduced for alignment. Identical amino acids are indicated by boxes. 2nd PP and LP are second mitochondrial processing peptidase and bacterial leader peptidase cleavage sites, respectively.

Following holo-p*LC<sub>I</sub>-c* formation, translocation of p*LC<sub>I</sub>-c* across the outer membrane is triggered thus drawing the protein across and in doing so the matrix-targeting domain gains access to the  $\Delta\Psi$ -dependent step and as a result translocation (at least of the presequence) across the inner membrane occurs. Cleavage by the MPP then ensues. Following heme addition, translocation of the presequence of p*LC<sub>I</sub>-c* across the inner membrane could occur at either one of two places. Either following holo-p*LC<sub>I</sub>-c* formation the p*LC<sub>I</sub>-c* folds and is released into the intermembrane space from where the presequence if still exposed, responds to the  $\Delta\Psi$  across the inner membrane and the presequence at least, inserts across the inner membrane whereby it becomes exposed to the matrix and the MPP. As it is unlikely that the folded cytochrome *c* would also cross the inner membrane, one would expect this holo-i*LC<sub>I</sub>-c* species to be peripherally located to the inner membrane yet exposed to the intermembrane space. A similar location was demonstrated for apo-i*LC<sub>I</sub>-c*, following its retranslocation across the inner membrane, but could not be demonstrated for holo-i*LC<sub>I</sub>-c*. The alternative is that the whole process is occurring at contact sites, preliminary evidence exists that suggests that CCHL is localized at contact sites (Nicholson & Neupert, in preparation) thus favoring this theory. Hence following heme addition, the p*LC<sub>I</sub>-c* protein, whilst still in the membrane system, gets drawn further across the outer membrane as the protein folds and the matrix-targeting sequence comes into the vicinity of contact sites and the  $\Delta\Psi$ -dependent steps of the translocation machinery and hence gains access to the matrix. It is unlikely that the protein finally accumulates in the matrix, as folding of the cytochrome *c* moiety following heme addition would probably serve to arrest the fusion protein in the contact site membrane system. Attempts to localize this holo-i*LC<sub>I</sub>-c* have not as yet conclusively demonstrated its sublocation, however it can be concluded that the protein is membrane-bound and does not appear to be greatly exposed either to the intermembrane space or the matrix compartments.

## 5.6 Membrane-insertion properties of apocytochrome *c* can substitute for receptor/GIP mediated steps of protein import.

The cytochrome *c* import pathway differs markedly from the general route taken by the majority of other imported proteins (which is characterized by the import involvement of namely, receptor/GIP, contact sites and by the requirement of  $\Delta\Psi$ ). Unique features of both the cytochrome *c* precursor (apocytochrome *c*) and of the mechanism that transports it into mitochondria have contributed to the evolution of a distinct import pathway that is not shared by any other mitochondrial protein analysed so far. The cytochrome *c* pathway is particularly unique because i) apocytochrome *c* appears to have spontaneous membrane insertion-activity (Rietveld *et al.*, 1983; Rietveld & de Kruijff, 1984; Rietveld *et al.*, 1985a, b, 1986; Dumont & Richards, 1984; Berkout *et al.*, 1987), ii) cytochrome *c* heme lyase seems to act as a specific binding site *in lieu* of a surface receptor (Nargang *et al.*, 1988; Nicholson *et al.*, 1988), and iii) covalent heme addition and the associated refolding of the polypeptide appears to provide the free energy for the translocation of the cytochrome *c* polypeptide across the outer mitochondrial membrane (Hennig & Neupert, 1981; Nicholson *et al.*, 1987; Nicholson *et al.*, 1988).

The ability of apocytochrome *c* to spontaneously insert into lipid bilayers has been the focus of research carried out by de Kruijff and his co-workers. They have demonstrated that the apocytochrome *c* can interact strongly, in an electrostatic manner, with the negatively-charged phospholipids in a model membrane system. This electrostatic interaction was thought to induce structural reorganization of the lipids leading to a reduction of the barrier function, and subsequently apocytochrome *c* penetrated into the bilayer giving rise to hydrophobic protein-lipid interactions. Circular dichroism (CD) studies revealed that the CD pattern of apocytochrome *c* in aqueous solution was featureless. However, penetration of the protein into the lipid bilayer was accompanied by the formation of  $\alpha$ -helical structures. Hence, it was postulated that this conformational change was important for the insertion of the protein into the lipid bilayer.

It has been previously suggested that a surface receptor does not appear to exist for cytochrome *c* import (Nicholson *et al.*, 1988). Instead, a partially inserted apocytochrome *c* forms a complex with cytochrome *c* heme lyase (the stage of high-

affinity binding to previously identified saturable binding sites, Hennig *et al.*, 1983) from which it reacts further to be converted to holocytochrome *c* and be translocated across the outer membrane. The membrane-insertion activity of apocytochrome *c* would appear, therefore, to be critical for enabling it to reach further specific components of the import pathway. As observed with pSc<sub>1</sub>-*c* and pLc<sub>1</sub>-*c*, the membrane insertion activity of apocytochrome *c* is not affected if the first six amino acids are removed and additional (foreign) sequences are added to the amino terminus. On the other hand, mitochondrial binding seems to be reduced if the carboxy terminus of apocytochrome *c* is modified (Stuart *et al.*, 1987) which suggests as concluded before that it is possible that a certain "folding state" might exist for membrane recognition and insertion of apocytochrome *c*.

It has been shown here that the membrane-insertion activity of apocytochrome *c* can aid the insertion of a mitochondrial targeting sequence attached to its amino-terminus in such a manner that the targeting sequence responds to  $\Delta\Psi$  and directs the fusion protein into the matrix. The presence of the apocytochrome *c* domain enables the pSc<sub>1</sub>-*c* hybrid protein to circumvent the receptor/GIP mediated steps of import into the matrix by directly mediating the insertion of the precursor protein into the mitochondrial membrane system where the targeting domain can then respond to the membrane potential for complete translocation across the inner membrane. This emphasizes the roles of receptors and GIP in membrane insertion in addition to the specific recognition of precursor proteins.

Taken together, these findings help explain why all other mitochondrial precursor proteins examined thus far require a receptor/GIP system for import. A precursor protein containing a target signal (i.e. a prepiece) but no membrane-insertion activity within the mature part of the polypeptide apparently depends on components in the outer membrane to recognize them and catalyse insertion of the targeting signal in such a manner that it can respond to  $\Delta\Psi$ . Because apocytochrome *c* is able to spontaneously insert into membranes it is able to mediate the circumvention of these critical stages of import on behalf of "passenger" sequences that would otherwise have to use a receptor and GIP in order to get to the  $\Delta\Psi$ -dependent step.

A series of research groups have claimed recently that sequences that are non-specific for mitochondrial protein import can, however, target passenger proteins to mitochondria (Hurt *et al.*, 1986; Allison & Schatz, 1986; Hurt & Schatz, 1987). At first glance it would appear that the mitochondrial protein import is less selective than previously imagined. However, as demonstrated by Pfaller *et al.*, 1989, import of such non-mitochondrial precursors does not require the presence of surface receptors and occurs with much lower efficiency and slower kinetics than the import of authentic mitochondrial precursor proteins (Pfaller *et al.*, 1989). Thus, this so-called "by-pass import" is not only less efficient but also less selective. Upon import into mitochondria, authentic precursor proteins (with the exception of apocytochrome c) must interact initially with specific receptors and then with GIP to become inserted into the mitochondrial membrane system. Pfaller *et al.*, 1988, have termed this a "multiple-check system" which controls the efficiency and specificity of precursor import at multiple stages of import. Hence *in vivo* such "by-pass import" would be so negligible that the uniqueness of the mitochondrial protein composition would not be threatened.

As demonstrated from the import studies of pLC<sub>1</sub>-c and pSc<sub>1</sub>-c, that if a fusion protein is constructed in such a manner that it artificially possesses membrane-insertion activity, in addition to a targeting sequence, it may display more efficient "by-pass import" into mitochondria because they can circumvent at least one step (the GIP-mediated) of this "multiple-check system" involved in mitochondrial protein import. This emphasizes the caveat for fusion proteins or modified (i.e. truncated) proteins which may artifactually possess membrane insertion activity and thereby bypass receptors (for a discussion see Pfanner *et al.*, 1988a).

## 5.7 Divergent evolution of import pathways for mitochondrial *c*-type cytochromes

Despite the number of functional similarities, the import pathways for the two mitochondrial *c*-type cytochromes (namely, cytochrome *c* and  $c_1$ ) as outlined in the Introduction are strikingly different. However, it appears that they have evolved from a common starting point. The primary amino acid sequence and three-dimensional X-ray crystallographic analyses of cytochromes from a diverse range of both mitochondria and bacteria have demonstrated that mitochondria are most likely to be descendants of purple photosynthetic bacteria, such as members of the *Rhodobacter* species.

Mitochondrial cytochrome *c* is very similar, both structurally and spectroscopically, to  $c_2$ -type bacterial cytochromes e.g. cytochrome  $c_2$  of *Rhodobacter capsulata*. Cytochrome  $c_2$  functions as an electron carrier in both the photosynthetic and mitochondrial chains of *R. capsulata*, although its presence in the cell is not essential for growth (Daldal *et al.*, 1986). The bacterial equivalent of the mitochondrial cytochrome  $c_1$  is also termed cytochrome  $c_1$  (e.g. cytochrome  $c_1$  from *R. sphaeroides*, Gabellini *et al.*, 1985). Like its mitochondrial equivalent, bacterial cytochrome  $c_1$  is an electron carrying subunit of the ubiquinol-cytochrome *c* reductase ( $bc_1$ ) complex. The bacterial cytochromes  $c_2$  and  $c_1$  are in many aspects very similar with regard to their biogenesis. Both cytochromes are synthesized as precursor proteins containing an amino-terminal leader sequence (Daldal *et al.*, 1986; Gabellini & Sebald, 1986). These leader sequences are very characteristic of bacterial leader sequences, namely, they are relatively short (each being only 21 residues), contain either one (cytochrome  $c_2$ ) or two (cytochrome  $c_1$ ) positive charges at the extreme amino terminus, followed by a very hydrophobic core rich in alanines and valine residues. The leader sequences of both cytochromes  $c_2$  and  $c_1$  are cleaved upon export of the proteins across the photosynthetic membrane to their functional location in the periplasm, a compartment corresponding to the mitochondrial intermembrane space, in that both are topologically opposed to the  $F_1$  part of the ATPase. As mentioned earlier these leader sequences of cytochromes  $c_2$  and  $c_1$  are very similar to the second domain of the mitochondrial cytochrome  $c_1$  presequence (Figure 37).

Following the endosymbiotic event, evolutionary gene transfer from the bacterial endosymbiont to the host cell nucleus occurred. Thus, the newly formed eucaryotic cell had to evolve new mechanisms to return the gene products to their functional location in the mitochondrion. Rather than completely reconstruct the means by which a cytoplasmically-synthesized mitochondrial protein was imported and assembled, the import pathways evolved so as to use evolutionary remnants of the pre-existing "ancestral assembly pathways" of the bacterial endosymbiont ("conservative sorting hypothesis", Hartl *et al.*, 1986; 1987). During evolution, however, the respective assembly pathways for cytochrome *c* and *c*<sub>1</sub> have diverged. Whereas cytochrome *c*<sub>1</sub> import into mitochondria has retained remnants of its "ancestral" assembly pathway, cytochrome *c* import occurs by a simpler and novel mechanism. How then has cytochrome *c* escaped the conservative sorting that cytochrome *c*<sub>1</sub> must follow during import ?

The membrane insertion properties of apocytochrome *c* may in part explain how the cytochrome *c* import pathway has evolved in such a way as to have escaped conservative sorting and receptor-mediated events. Spontaneous insertion into mitochondrial membrane may then be the first step in cytochrome *c* translocation. There may be no specificity of this step for the mitochondrial outer membrane or a limited specificity (e.g. exerted by the particular lipid composition). Only in the case of the mitochondria, however, is there a specific recognition and tight binding involving cytochrome *c* heme lyase. This then appears to be followed by the covalent heme attachment and then folding-dependent translocation across the outer membrane accounts for the absence of the requirement for a surface receptor and GIP during import. In contrast, because the mature part of cytochrome *c*<sub>1</sub> does not have membrane insertion activity, both the receptor and GIP are required for specific recognition and membrane insertion. Once initiated along this route, cytochrome *c*<sub>1</sub> then apparently requires transit along a conservative sorting pathway (i.e. via the matrix) in order to become properly assembled in the intermembrane space. It is postulated here therefore, that the unique property of apocytochrome *c* to spontaneously insert into membranes confers upon the precursor the ability to circumvent the early classical steps of mitochondrial protein import, namely, surface receptor binding and initial membrane insertion mediated by GIP.

In summary, the striking difference in the mechanisms by which cytochromes *c* and *c*<sub>1</sub> are imported into the intermembrane space thus reflects the divergence of their assembly pathways during the evolution of mitochondria. It is likely that the major factor contributing to this divergence is the difference in their respective membrane insertion properties.

## 6. SUMMARY

Molecular cloning and characterization of cytochrome *c* cDNA of *Neurospora crassa* wild-type and a cytochrome *c*-deficient mutant (*cyc1-1*) are described. Southern blot analysis of genomic DNA indicates that only one gene exists per haploid genome. The cDNA sequence of the wild-type gene confirmed the previously determined protein sequence. Sequence analysis of the *cyc1-1* cDNA revealed the presence of a larger open reading frame, owing to the presence of an unspliced intron at the 3' end of the coding region. Splicing of this intron is obviously retarded due to the presence of two base exchanges in the highly conserved intron consensus sequences. Consequently, *cyc1-1* synthesizes an apocytochrome *c* with an altered carboxy terminus, 19 amino acids longer than the wild-type, with the final 27 amino acids being of an unrelated sequence. This alteration in the carboxy terminus renders the apocytochrome *c* incompetent for binding to mitochondria and consequently, import into mitochondria. Apocytochrome *c* is particularly unique amongst mitochondrial precursor proteins because it has membrane-insertion activity which enables it to spontaneously penetrate the outer mitochondrial membrane. The addition of extra amino acids at the carboxy terminus of apocytochrome *c* does not significantly reduce its membrane-insertion activity, instead specific binding of *cyc1-1* apocytochrome *c* to cytochrome *c* heme lyase (CCHL) would appear to be severely hindered. These results suggest that the specific binding of cytochrome *c* to CCHL is mediated via its carboxy terminus.

The problem of how other precursor proteins initially become inserted into mitochondrial membranes was also investigated by examining the import mechanism of hybrid proteins which contain dual targeting information and, at the same time, membrane insertion activity. The hybrid protein pSc<sub>1</sub>-*c* is comprised of the matrix-targeting domain of the cytochrome *c*<sub>1</sub> presequence joined to the amino terminus of apocytochrome *c*. pSc<sub>1</sub>-*c* could be selectively imported along either a cytochrome *c*<sub>1</sub> route into the mitochondrial matrix or via a cytochrome *c* route into the intermembrane space. pSc<sub>1</sub>-*c* initiated on the cytochrome *c* import pathway could be redirected to a route leading to the matrix, demonstrating that a functional intersection between these distinct import mechanisms exists. In contrast to cytochrome *c*<sub>1</sub>, pSc<sub>1</sub>-*c* did not require the receptor /GIP (general

insertion protein) system for entry into the matrix. It may be concluded that the apocytochrome *c* domain in the fusion protein could exert its membrane insertion activity in such a manner that the matrix-targeting sequence gained direct access to the membrane potential-dependent step and as a result triggered translocation across the inner mitochondrial membrane. These observations emphasized, in addition to the function of recognition and binding precursors, the important role of the receptor/GIP system in facilitating the initial insertion of precursors into the mitochondrial membranes.

A related protein, p*Lc<sub>1</sub>-c*, comprised of the entire cytochrome *c*<sub>1</sub> presequence joined to the amino terminus of apocytochrome *c*, was also constructed. Like p*Sc<sub>1</sub>-c*, p*Lc<sub>1</sub>-c* could also be imported into mitochondria along both routes in a manner which was independent of surface receptors and GIP. It was directly shown from the results of the import p*Lc<sub>1</sub>-c* fusion protein that the cytochrome *c*<sub>1</sub> presequence alone contains all the necessary information for intramitochondrial targeting and that the hydrophobic domain of the presequence acts as a "re-export" sequence directing the export of the matrix localized i*Lc<sub>1</sub>-c* across the inner membrane into the intermembrane space.

The p*Sc<sub>1</sub>-c* and p*Lc<sub>1</sub>-c* fusion proteins, in contrast to other precursors, did not require nucleotide triphosphates to be imported into the matrix. Thus like apocytochrome *c*, it was demonstrated that a more folded conformation of the precursor is more membrane active and hence, is favoured in order to be translocation-competent.

It is proposed here that the presence of the membrane-insertion activity of apocytochrome *c* in both p*Sc<sub>1</sub>-c* and p*Lc<sub>1</sub>-c* confers upon their precursors the ability to circumvent the early classical steps of mitochondrial protein import, namely, ATP-dependent unfolding, surface receptor binding and initial membrane insertion mediated by GIP. It is likely that a major factor contributing to the evolutionary divergence observed in the assembly pathways of cytochrome *c* and cytochrome *c*<sub>1</sub> is this membrane active nature displayed by apocytochrome *c*; this property enables the cytochrome *c* precursor to escape the conservative sorting that cytochrome *c*<sub>1</sub> must follow in order to gain access to the mitochondrial intermembrane space.

## 7. ABBREVIATIONS

|                     |                                             |
|---------------------|---------------------------------------------|
| AMPS:               | Ammonium peroxydisulphate                   |
| ADP:                | Adenosine diphosphate                       |
| ATP:                | adenosine triphosphate                      |
| ATPase:             | adenosine triphosphatase                    |
| bp:                 | base pairs                                  |
| BSA:                | bovine serum albumin                        |
| CDTA:               | 1,2-cyclohexylenedinitrolo tetraacetate     |
| CCHL:               | cytochrome <i>c</i> heme lyase              |
| CC <sub>1</sub> HL: | cytochrome <i>c</i> <sub>1</sub> heme lyase |
| cpm:                | counts per minute                           |
| CTP:                | cytosine triphosphate                       |
| dATP:               | deoxyadenosine triphosphate                 |
| dCTP:               | deoxycytosine triphosphate                  |
| dGTP:               | deoxyguanosine triphosphate                 |
| dTTP:               | deoxythymidine triphosphate                 |
| dNTP:               | deoxynucleotide triphosphate                |
| ddATP:              | dideoxyadenosine triphosphate               |
| ddCTP:              | dideoxycytosine triphosphate                |
| ddGTP:              | dideoxyguanosine triphosphate               |
| ddTTP:              | dideoxythymidine triphosphate               |
| DMSO:               | dimethyl sulphoxide                         |
| DNase:              | deoxyribonuclease                           |
| DNA:                | deoxyribonucleic acid                       |
| DTT:                | dithiotreitol                               |
| EDTA:               | ethylenediamine tetraacetate                |

|           |                                                        |
|-----------|--------------------------------------------------------|
| EGTA:     | ethyleneglycol-bis-aminoethylether tetraacetate        |
| HEPES:    | N-2 hydroxyethyl piperazine-N'-2-ethane sulphonic acid |
| HPLC:     | high performance liquid chromatography                 |
| hsp:      | heat shock protein                                     |
| kD:       | kilo Dalton                                            |
| LB:       | Luria broth                                            |
| MES:      | 2-[N-Morpholino]ethane sulphonic acid                  |
| min:      | minutes                                                |
| Mops:     | N-morpholinopropane sulphonic acid                     |
| MPP:      | matrix processing peptidase                            |
| mRNA:     | messenger ribonucleic acid                             |
| MW:       | molecular weight                                       |
| NADH:     | nicotineamide adenine dinucleotide                     |
| OD:       | optical density                                        |
| PEG:      | polyethyleneglycol                                     |
| PEP:      | processing enhancing protein                           |
| PMSF:     | phenylmethysulphonylfluoride                           |
| PVP:      | polyvinylpyrrolidone                                   |
| RNase:    | ribonuclease                                           |
| RNasin:   | ribonuclease inhibitor                                 |
| SDS:      | sodium dodecyl sulphate                                |
| SDS/PAGE: | SDS-polyacrylamide gel electrophoresis                 |
| TEMED:    | N,N,N',N'-tetramethylethylenediamine                   |
| Tris:     | tris(hydroxymethyl)aminomethane                        |
| UTP:      | uridine triphosphate                                   |

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## Acknowledgments

I would like to take this opportunity to express my sincere gratitude to Professor Dr. Dr. Walter Neupert for all his help and encouragement throughout my stay in the Institut für Physiologische Chemie.

I wish also to express my thanks to both Dr. Max Tropschug and Dr. Don Nicholson for all their advice, encouragement and above all, kindness shown to me over the past years.

A special thanks also goes to Christine Kraus for all her assistance and her photographic expertise.

To all my friends and co-workers, especially Dr. Rupert Pfaller, Professor. Dr. Richard Zimmermann, Christoph Hergersberg, Michael Arretz, Dr. Ulrich Hartl, Heinrich Steger, Joachim Rassow, Sigrun Meier, Kerstin Malke, Hans Koll, Dr. Günter Müller, and Dr. Gerd Hawlitschek, I am very grateful, not only for their help and advice, but also for the many good times and laughs we shared together. They will always be remembered.

I would also like to take the opportunity to thank the Deutscher Akademischer Austauschdienst who very generously sponsored my stay here in Munich from 1985-1988.

Finally, but most importantly, no amount of words could express the depth of my gratitude to my parents for their constant support and encouragement shown to me over the past years.

I dedicate this work, therefore, both to my mother and to the memory of my late father.

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